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Induction of diabetes in Wistar rats: is the streptozotocin model feasible at any age?

Indução de diabetes em ratos Wistar: o modelo da estreptozotocina é válido em qualquer idade?

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ABSTRACT
Diabetes mellitus (DM) is an epidemic that affects millions of people worldwide. Because of its high morbidity and mortality, animal models of diabetes are indispensable to achieve a better understanding of the pathophysiological mechanisms and discover new treatments. The classic used model to induce type 1 DM involves streptozotocin (STZ) administration and it is well known that the association of streptozotocin with a high fat diet also induces type 2 DM. However, these studies are performed in rats between 60-90 days and should be performed in older animals because aging is a major risk factor for other diseases, that occur concomitantly with DM. Thus, this study evaluated diabetes in aged Wistar rats at 300 days. Diabetes was induced by an intraperitoneal injection of 60 mg/kg STZ. Three days after DM induction, the rats exhibited alterations in clinical signs and increases in the levels of aspartate and alanine aminotransferase, urea, and creatinine. Microscopic changes were observed in the kidneys and liver. These findings indicate that this model is not feasible in rats at 300 days of age and should not be attempted by other research groups or should be performed by researchers who want to study the complications of diabetes.

Keywords: Animal model, Drug-induced toxicity, Hyperinsulinemia, Preclinical studies, Toxicity.

RESUMO
O diabetes mellitus (DM) é uma epidemia que afeta milhões de pessoas em todo o mundo. Devido à sua alta morbimortalidade, os modelos animais de diabetes são indispensáveis para se compreender melhor os mecanismos fisiopatológicos e descobrir novos tratamentos. O modelo clássico usado para induzir DM tipo 1 envolve a administração de estreptozotocina (STZ) e é sabido que a associação de estreptozotocina com dieta rica em gordura também induz DM tipo 2. No entanto, esses estudos são realizados em ratos entre 60 a 90 dias e devem ser realizados em animais mais velhos, pois o envelhecimento é um fator de risco importante para outras doenças, que ocorrem concomitantemente ao DM. Assim, este estudo avaliou o diabetes em ratos Wistar idosos aos 300 dias. O diabetes foi induzido por uma injeção intraperitoneal de 60 mg / kg de STZ. Três dias após a indução do DM, os ratos exibiram alterações nos sinais clínicos e aumento dos níveis de aspartato e alanina aminotransferase, uréia e creatinina. Alterações microscópicas foram observadas nos rins e fígado. Esses achados indicam que esse modelo não é viável em ratos com 300 dias de idade e não deve ser tentado por outros grupos de pesquisa ou deve ser realizado por pesquisadores que desejam estudar as complicações do diabetes.

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Palavras-chave: Modelo animal, Toxicidade induzida por drogas, Hiperinsulinemia, Estudos pré-clínicos, Toxicidade.

1 INTRODUCTION

Diabetes mellitus is considered a worldwide epidemic, affecting ~415 million people. This number is estimated to reach more than 600 million by 2040 (IDF, 2015). Brazil has an estimated 13 million diabetic patients, ranking it fourth worldwide (IDF, 2015). Approximately 320 million individuals in the world have low glucose tolerance, which can lead to the development of diabetes (IDF, 2015).

Diabetes mellitus has a high economic impact. The utilization of financial healthcare resources is two- to three-times higher for diabetic patients than for healthy individuals. Approximately 12% of the world's healthcare expenditures are dedicated to diabetes and its complications, including cerebrovascular disease, retinopathy, neuropathy, nephropathy, and cardiovascular disease (ALBERTI; ZIMMET, 1998; VINIK et al., 1992; KLEIBERGER; POLLIN, 2015). These complications are the main cause of morbidity and mortality in this group of patients. Such complications can occur because of the greater permeability of certain tissues to circulating glucose, making them proportionally more affected by occasional or persistent hyperglycemia (KLEIBERGER; POLLIN, 2015; MAKRIS; SPANOU, 2015).

Because of its high morbidity and mortality, animal models of diabetes are indispensable to achieve a better understanding of the pathophysiological mechanisms and develop more effective drugs with fewer side effects. The classic and widely used model to induce diabetes in rodents involves the administration of streptozotocin (STZ), which is derived from *Streptomyces achromogenes*. Streptozotocin is clinically used for the treatment of pancreatic β-cell carcinoma. A 60 mg/kg dose of STZ can destroy Langerhans islet β-cells and lead to clinical diabetes within 2-4 days (SZKUDELSKI, 2001; LENZEN, 2008).

The model of STZ-induced diabetes was proposed in 1963 by Rakieten and Nadkarni (1963) and has been utilized extensively in preclinical research. Traditionally, STZ is used to induce type 1 diabetes mellitus but Reed et al. (2000) proposed a rodent model for type 2 diabetes through the association of f high fat diet and streptozotocin administration. The vast majority of these studies have been performed in rats between 60 and 90 days of age. However, diabetes is a disease that should be investigated in older animals because aging is a major risk factor for other diseases, such as cardiovascular disease, that occur concomitantly with

diabetes. Thus, the aim of the present study was to evaluated diabetes induction by STZ in rats at 300 days of age.

2 MATERIAL AND METHODS

2.1 ANIMALS

Diabetes induction was performed in male Wistar rats, weighing 400-600 g and 10 months old, that were obtained from the State University of Maringá (UEM). The animals were housed (16 animals divided in 4 animals per cage) in the laboratory of Preclinical Research of Natural Products of Paranaense University (UNIPAR) with free access to food and water under controlled environmental conditions (temperature: 20°C ± 2°C; relative humidity: 50% ± 10%; 12 h/12 h light/dark cycle). The animals were kept in polypropylene boxes (49 x 34 x 16 cm - length x width x height), with shavings as bedding, environmental enrichment and transfer to clean boxes every day. The experimental protocol was approved by the Ethics and Research Committee on Animals of UNIPAR (authorization no. 34676/2018) and was performed in accordance with international standards and ethical guidelines on animal welfare, including The Guide to the Care and Use of Experimental Animals from Canadian Council on Animal Car. The reduction of the number of animals that are used for research purposes.

2.2 DIABETES INDUCTION AND BLOOD GLUCOSE MEASUREMENT

For diabetes induction, after a 12-h fast, the animals in the diabetic group (n = 8) received 60 mg/kg STZ (i.p.) diluted in 10 mM citrate buffer (pH 4.5). The animals in the normoglycemic group (n = 8) received only vehicle (1 ml/kg citrate buffer, i.p.). After the injection, the rats were fasted for an additional 1 h to avoid competition of the drug with glucose molecules from the diet, thus increasing the chances of binding to glucose transporters (GLUT) receptors on β-pancreatic cells. Three days after STZ administration, a small volume of peripheral blood was collected from the tail and placed on reactive tapes. Blood glucose levels were measured using Accu Check Active (Roche). Rats were considered diabetic when glycemia was ≥ 250 mg/dL.

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2.3 MONITORING OF ANIMALS

After diabetes induction, the rats were examined daily for 3 days by a veterinarian for signs of systemic toxicity, such as palpebral ptosis, piloerection, salivation, anorexia, diarrhea, tachypnea, apathy, prostration, lower responsiveness to touch, and ataxia.

2.4 BIOCHEMICAL ANALYSIS

For the evaluation of biochemical parameters, 3 days after diabetes induction, the rats were anesthetized in a chamber that was saturated with isoflurane (1-3%), and blood was collected from the ocular plexus. Plasma levels of aspartate aminotransferase (AST; U/L), alanine aminotransferase (ALT; U/L), creatinine (mg/dl), and urea (mg/dl) were determined using an automated enzymatic colorimetric method.

2.5 EUTHANASIA AND SAMPLE COLLECTION

After blood collection, the rats were placed again in the chamber that was saturated with isoflurane for euthanasia by deep anesthesia. Afterward, samples of the kidneys, bladder, liver and spleen were collected, fixed in 10% formaldehyde, sectioned, and stained with hematoxylin and eosin. Cellular changes that resulted from diabetes induction were evaluated by a veterinarian pathologist under an optical microscope (LEICA DM 2500).

2.6 STATISTICAL ANALYSIS

The data were analyzed using Student's *t*-test and are expressed as mean and standard error of mean. Values of $p < 0.05$ were considered statistically significant. GraphPad Prism 5.0 software was used for the statistical analysis and generation of the graphs.

3 RESULTS

3.1 GLYCEMIA LEVELS AFTER STREPTOZOTOCIN ADMINISTRATION

Before the administration of STZ, the glucose levels of rats were 107.30 ± 2.95 mg/dL. As expected, STZ administration in 300-day-old rats significantly increased glucose levels by 445.97% compared with the basal group (109.20 ± 3.18 mg/dL; Fig. 1).

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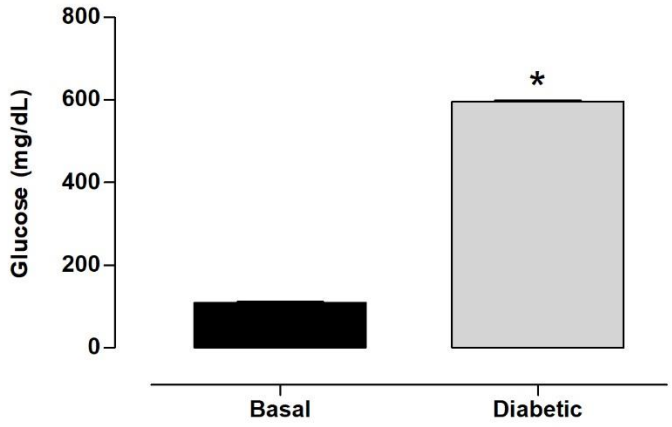
Figure 1. Plasma levels of glucose. Rats received vehicle (Basal group, *n* = 8) and streptozotocin (Diabetic group, *n* = 8). The results are expressed as mean ± SEM. **p* < 0.05, *vs.* Basal group (Student's *t*-test).



3.2 STREPTOZOTOCIN INDUCES ALTERATIONS OF CLINICAL SIGNS IN RATS

No significant changes in clinical signs were observed in the basal group. In the diabetic group, 3 days after diabetes induction, the rats exhibited palpebral ptosis, piloerection, anorexia, diarrhea, tachypnea, apathy, prostration, and lower responsiveness to touch compared with normoglycemic rats.

3.3 STREPTOZOTOCIN INCREASES PLASMA BIOMARKERS OF HEPATIC INJURY IN RATS

Streptozotocin administration in 300-days-old rats significantly increased AST levels (209.3 ± 33.08 U/L) by 95.32% compared with the basal group (107.0 ± 12.29 U/L; Fig. 2A) and increased ALT levels (98.00 ± 12.38 U/L) compared with the basal group (69.17 ± 6.90 U/L; Fig. 2B).

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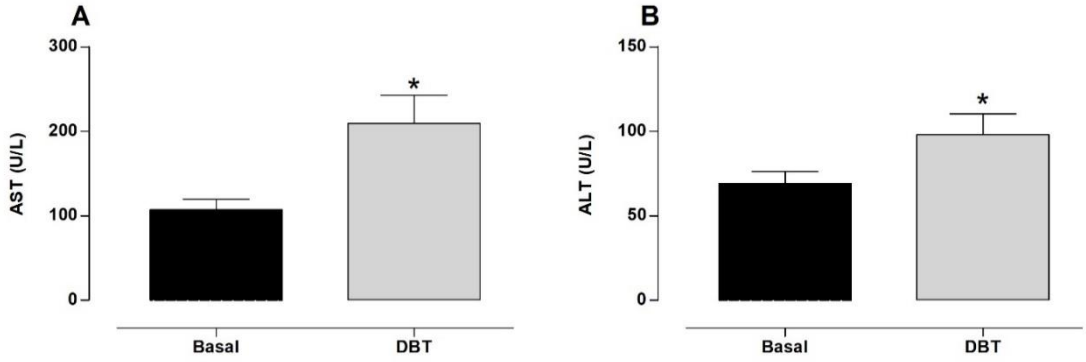
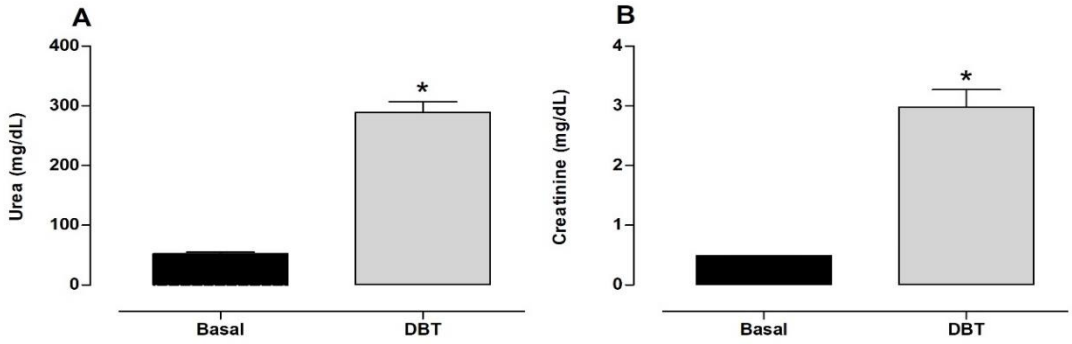
Figure 2. Plasma levels of hepatic damage markers. Levels of (A) aspartate aminotransferase and (B) alanine aminotransferase in rats that received vehicle (Basal group, $n = 8$) and streptozotocin (Diabetic group, $n = 8$). The results are expressed as mean ± SEM. *$p < 0.05$, *vs.* Basal group (Student's *t*-test).



3.4 STREPTOZOTOCIN INDUCES RENAL LESIONS IN RATS

With regard to serum levels of biomarkers of renal injury, 455.76% and 504.16% increases in urea (289.2 ± 17.82 mg/dL; U/L; Fig. 3A) and creatinine (2.97 ± 0.29 mg/dL; Fig. 3B), respectively, were observed in diabetic rats compared with the basal group (52.12 ± 2.83 mg/dL and 0.48 ± 0.50 mg/dL, respectively).

Figure 3. Plasma levels of renal damage markers. Levels of (A) urea and (B) creatinine in rats that received vehicle (Basal group, $n = 8$) and streptozotocin (Diabetic group, $n = 8$). The results are expressed as mean ± SEM. *$p < 0.05$, *vs.* Basal group (Student's *t*-test).



3.5 HISTOPATHOLOGICAL ANALYSIS

Diabetes induction in 300-day-old rats also caused microscopic changes. The moderate to severe presence of hyaline cylinders was observed in the cortical region, with accentuated and diffuse cytoplasmic vacuolization of the tubular epithelium, suggesting glycogen deposition in the kidneys in diabetic rats (Fig. 4B) compared with normoglycemic rats (Fig. 4A). With regard to hepatic alterations, diabetes induction caused marked hepatic glycogen

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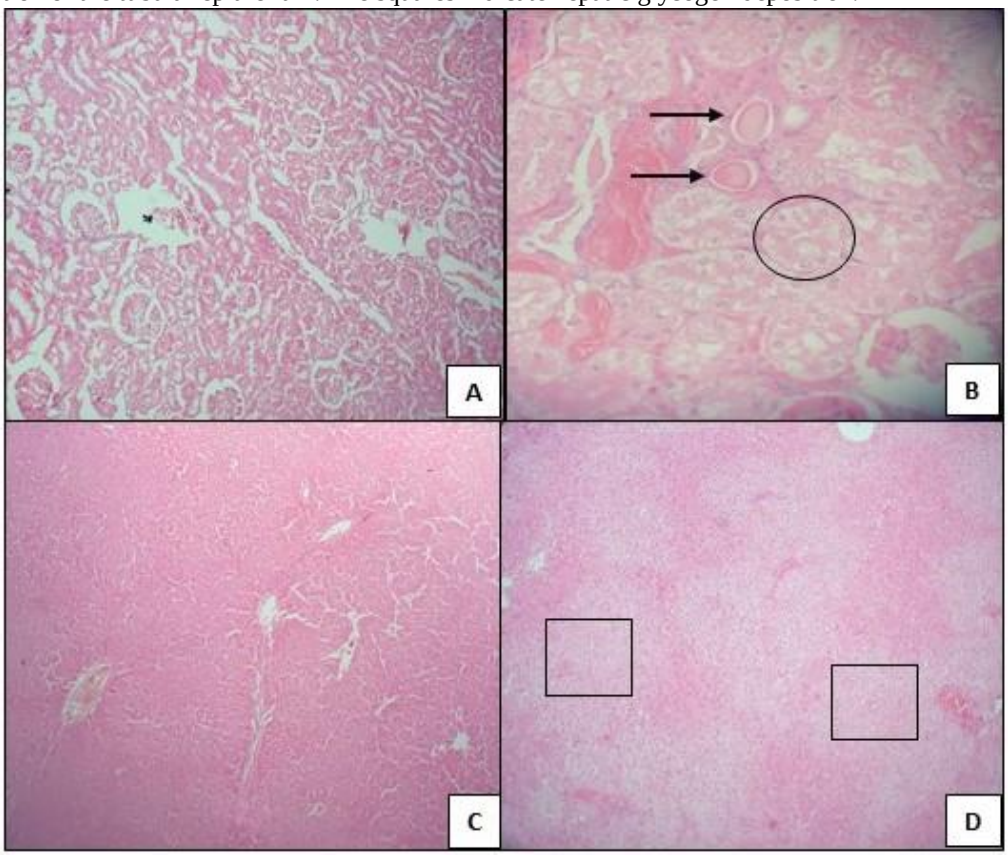
deposition, from multifocal to coalescent (Fig. 4D), compared with normoglycemic animals (Fig. 4C).

Figure 4. Kidney and hepatic histopathological evaluation. (A, B) Histopathological analyses of the kidneys in rats that received (A) vehicle (Basal group, *n* = 8) and (B) streptozotocin (Diabetic group, *n* = 8). (C, D) Histopathological analyses of the liver in rats that received (C) vehicle (Basal group, *n* = 8) and (D) streptozotocin (Diabetic group, *n* = 8). Arrows indicate hyaline cylinders in the cortical region. The circle indicates cytoplasmic vacuolization of the tubular epithelium. The squares indicate hepatic glycogen deposition.



4 DISCUSSION

Streptozotocin-induced diabetes is a classic model that is simple, accessible, and valid. Hundreds of research groups have investigated the pathophysiological mechanisms of this disease and evaluated the antidiabetic potential of drugs using this model for type 1 and type 2 diabetes mellitus (REED et al., 2000; ARULMOZHI; VEERANJANEYULU; BODHANKAR, 2004; SRINIVASAN et al., 2005; AKBARZADEH et al., 2007; FURMAN, 2008; MANSOR et al., 2013; CASTRO et al., 2020). However, to date, this method of diabetes induction has been conducted in young adult rats, 60-90 days of age. The present study investigated this model of diabetes induction in older rats, with the goal of extrapolating the use of this model to investigate cardiovascular disease because both age and diabetes mellitus

are risk factors for the development of cardiovascular disorders. After administration of STZ, the animals would get a high fat diet for type 2 diabetes induction. However, because of the advanced age of the animals (300 days), we could not standardize this animal model because the animals developed clinical and laboratory alterations that indicated renal and hepatic insufficiency.

Animal models have historically played a critical role in understanding and characterizing the pathophysiology of various diseases and identifying possible targets for the development of new therapeutic agents (MCGONIGLE; RUGGERI, 2014). An ideal animal model should (*i*) allow the study of biological phenomena or animal behavior, (*ii*) allow the investigation of a spontaneous or induced pathological process, and (*iii*) reproduce, in one or more aspects, the phenomenon that occurs in humans (ANDERSEN; WINTER, 2017). The model of diabetes induction by STZ should be reproduced in animals with other associated risk factors, such as age because this is a risk factor that occurs concomitantly with diabetes. However, in the present study, when we sought to reproduce this model in older animals, we observed high morbidity, thus indicating that this model is not feasible in rats of advanced age.

Since the "3 Rs" (Reduction, Refinement, and Replacement) were introduced by Russell and Burch (1959), the scientific community has committed itself to reducing the number of animals that are used in research, improving experimental designs, minimizing animal suffering, and searching for alternative methods that can ultimately replace *in vivo* testing (FRANCO; OLSSON, 2014). Therefore, the present findings are important because they can inform researchers to not repeat these experiments, thus reducing the number of animals that are used for experimentation.

The publication of negative results is an essential element for the evolution of science because they can indicate what should not be reproduced. Such results are valuable clues that can become a basis for developing new hypothesis and designing new experiments that can then allow testing with a more restricted number of variables (SILVA, 2015). Nonetheless, the publication of unexpected or negative results can entail extra difficulties for the researcher. Many researchers emphasize, explore, and discuss positive outcomes, failing to document negative outcomes that are considered insignificant (OBERHOFER; LENNON, 2014). Although this attempt to standardize a model of STZ-induced diabetes in older rats was unsuccessful, these results are important because they provide additional insights into this subject, thus saving time and resources and preventing other research groups from attempting to replicate the same experiment.

In conclusion, the model of diabetes induction by STZ was not feasible in 300-day-old rats and should not be replicated by other research groups to avoid the unproductive use of animals or should be performed only by researchers who want to study the complications of diabetes in older animals.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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