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Pharmacological induction of diabetes mellitus in pregnant female mice: a comparison of two doses and routes of administration

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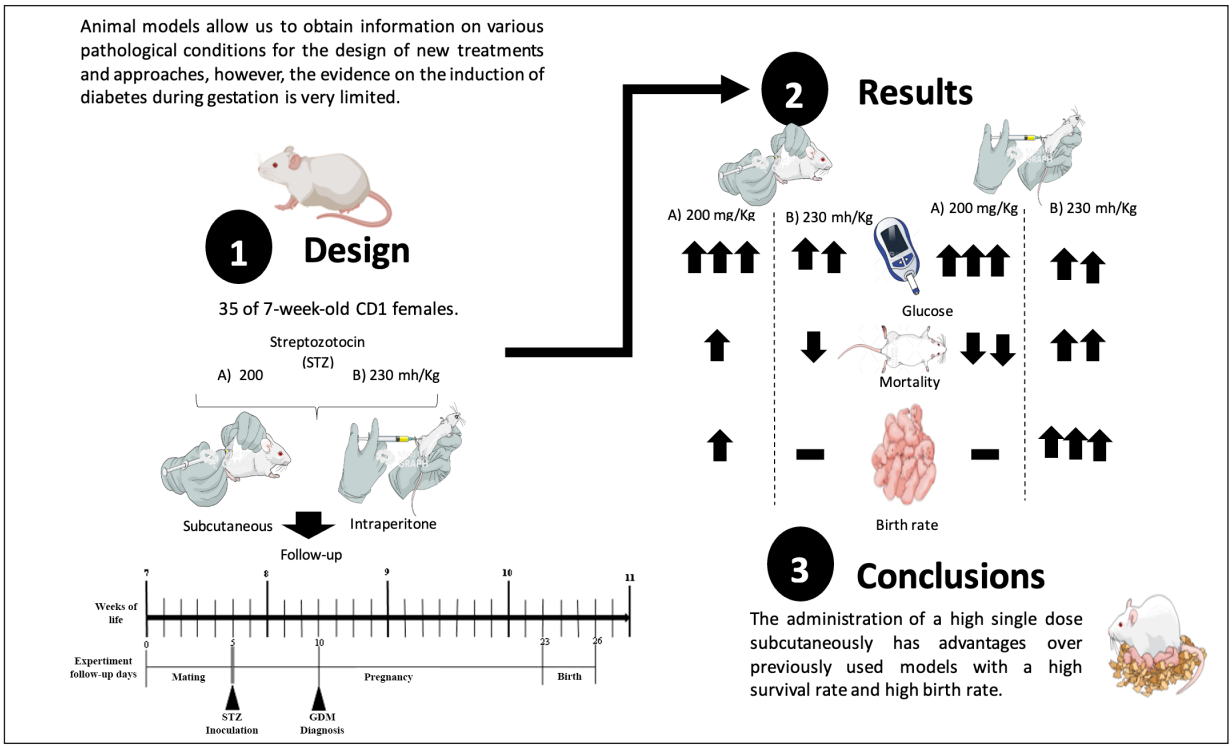
Abstract. – OBJECTIVE: This study aimed to compare two routes of administration and different dosages of streptozotocin (STZ) for the pharmacological induction of gestational diabetes mellitus (GDM) in pregnant CD1 females.

MATERIALS AND METHODS: 35 female CD1 mice were divided into 5 groups (n = 7). Diabetes mellitus (DM) was induced with STZ by two routes and two doses: 1) Control Group without administration of STZ (CL), 2) Intraperitoneal



Graphical Abstract. Pharmacological induction of diabetes mellitus in pregnant females: comparison of two doses and routes of administration.

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Group with 200 mg of STZ/Kg of weight (IP200), 3) Intraperitoneal Group with 230 mg of STZ/Kg of weight (IP230), 4) Subcutaneous Group with 200 mg of STZ/Kg of weight (SC200) and 5) Subcutaneous Group with 230 mg of STZ/Kg of weight (SC230). Body weight, food and water intake, glycemia, Homeostatic Model Assessment of Insulin Resistance Index (HOMA-IR), survival, and birth rate were identified.

RESULTS: **The SC230 group turned out to be the most effective dose and route for the induction of GDM in pregnant females. This scheme managed to reproduce sustained hyperglycemia with high HOMA-IR, the presence of polyphagia, polydipsia, and weight loss. In addition, the birth rate and survival were high compared to the other doses and routes of administration.**

CONCLUSIONS: **The administration of a single dose of 230 mg/kg of weight by subcutaneous route supposes advantages compared to previously used models since it decreases the physiological stress due to manipulation and the costs since it does not require repeated doses or adjuvants such as high lipid diets to potentiate the diabetogenic effect of STZ.**

Key Words:
Gestational diabetes mellitus, Pharmacology induction, Streptozotocin, Insulin resistance, Survival.

Introduction

Diabetes mellitus (DM) is a metabolic chronic disease that affects millions of people around the world[1]. It is characterized by a sustained hyperglycaemic state in the blood due to deficient secretion or altered action of insulin by the pancreas[2]. The most common forms of DM are type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM)[3]. However, there is another form of diabetes that can occur in pregnant women: gestational diabetes mellitus (GDM). This form of diabetes can have negative effects in the second and third trimesters of pregnancy[4], and it is characterized by impaired carbohydrate metabolism[5] and can spontaneously resolve itself at the end of pregnancy[6]. Gestational diabetes mellitus leads to insulin resistance (IR) that is attributed, in part, to the production of placental hormones. It is also characterized by glucose intolerance and an increase in pro-inflammatory cytokines such as Tumour Necrosis Factor-alpha (TNF-α) and Interleukin-6 (IL-6)[7,8]. GDM mainly affects women who have a body mass index (BMI) $\geq$ 24.9 kg/m^2 prior to pregnancy[9]. However, women with a normal BMI but multiple risk factors (e.g., genetic, environmental, maternal age equal to or greater than 25 years, multiparity, a family history of T2DM, a personal history of GDM in previous pregnancies, excessive weight gain during pregnancy, dyslipidemia, a sedentary lifestyle, and poor eating habits) are more likely to develop GDM[7,9,10]. It is also associated with multiple complications in the fetus[4,8] and pregnant women[11].

Animal models are valuable for obtaining information about pathological conditions and new treatments[12]. Pancreatectomy was the first animal model of diabetes used to study the central role of the pancreas in glucose regulation and insulin purification[13-15]. Diabetes mellitus has been successfully induced in mice, rats, rabbits, monkeys, cats, and dogs[16]. There are different models of induction: chemical, surgical, and genetic manipulation[17]. Within chemical methods, the most widely used drugs are alloxan and streptozotocin (STZ), which are glucose analogues[18]. Streptozotocin is an antimicrobial agent that is alkylating[19], with a high affinity for the glucose transporter 2 (GLUT2), which it uses to permeate the cell[20]. Once inside the cell, STZ accumulates in the cytosol and forms diazomethane, which induces deoxyribonucleic acid (DNA) alkylation, which in turn leads to death and selective destruction of pancreatic beta cells[16,21], that results in deficient insulin secretion or deficient action in tissues[22,23] that trigger hyperglycemia and IR[20,24]. In addition, the production of free radicals (e.g., superoxide and hydrogen peroxide) increases, which interrupts the production of adenosine triphosphate (ATP) at the mitochondrial level *via* poly ADP-ribosylation (poly adenosine diphosphate-ribosylation), reducing intracellular oxidized form of nicotinamide adenine dinucleotide (NAD^+)[12,25]. Streptozotocin largely relies on an intraperitoneal route, with doses between 40 and 200 mg/kg[19]. Some studies[26-28] have associated a high-fat diet with STZ administration to potentiate the hyperglycemic effect of the drug. However, very few murine studies[29] have been carried out in pregnant females, and the majority of them have induced DM in early stages after weaning. The dose of STZ has varied: in female Wistar rats, two doses have been administered: 100 mg/kg and 135 mg/kg *via* an intraperitoneal or subcutaneous route at 2 and 5 days of life with intermittent fasting lasting from 4-8 hours[30]. Two different doses of STZ have been used in female mice: 40 mg/kg for 4 or 5 days and a single dose of 200-230 mg/kg intraperitoneally. In both cases, STZ was combined with a 4-hour fast, followed by a high-fat diet that was initiated two weeks prior to STZ administration[26-28]. Although there is a wide variety of DM induction

methods relevant to female rats and mice of reproductive age, evidence pertaining to the induction of DM during pregnancy in murine models remains limited[29-34]. Most of the studies that have evaluated the impact of hyperglycemia on fertility, placental morphological changes[35-39], and the effects on the offspring of mothers with DM have relied on intraperitoneal induction of DM prior to mating with the support of other factors such as high-fat diets or fasting prior to induction[21,27,28,34,39-41]. As a result, the objective of this study was to compare two routes of administration and two different doses of STZ to induce GDM without added factors such as fasting, food restriction, or a high-fat diet.

Materials and Methods

Study Design

The experimental study consisted of 35 CD1, 7-week-old, pathogen-free female mice. The animals were kept and handled at the vivarium of the Faculty of Medicine, Universidad Autónoma del Estado de México, under the guidelines established by the Official Mexican Standard NOM-062-ZOO-199 Technical specifications followed the production, care, and use of laboratory animals and the Animal Research: Reporting of In Vivo Experiments (ARRIVE) standards for animal studies[42]. The protocol was approved by the Faculty Research Ethics Committee (CONBIOETICA-15-CEI-002--20210531). The mice were fed a standard normal diet [Rodent Laboratory Chow 5001 from Purina (3.02 Kcal/g)] and offered water *ad libitum*. Two females per cage were housed under controlled conditions of 19-21°C with 12-hour light/dark cycles.

Study Groups

The mice were randomly divided into five groups of seven animals each. Two routes of administration and two different doses of STZ were used: 1) Control Group: no drug administration (CL), 2) Intraperitoneal Group: STZ administered at 200 mg/kg (IP200), 3) Intraperitoneal Group: STZ administered at 230 mg/kg (IP230), 4) Subcutaneous Group: STZ administered at 200 mg/kg of weight (SC200), and 5) Subcutaneous Group: STZ administered at 230 mg/kg (SC230).

Induction of Gestational Diabetes Mellitus

This study proposes that DM can be induced simultaneously with gestation. Two females were placed for mating with one male five days prior to the induction of DM with STZ. The presence of a

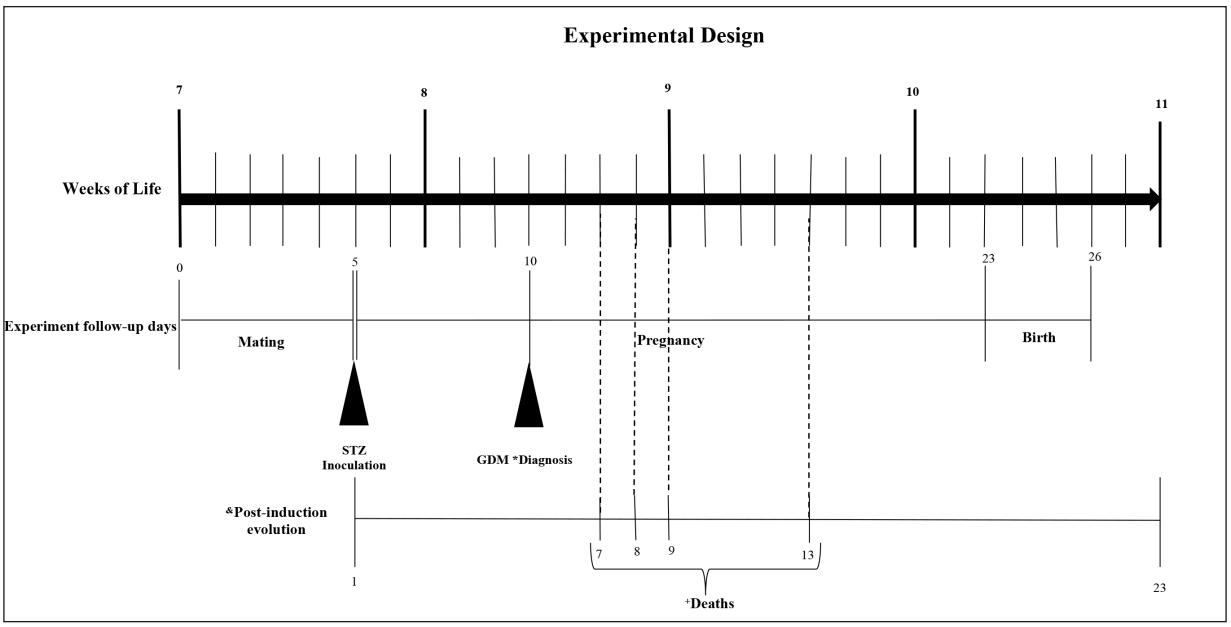

Figure 1. Experimental design for induction of Gestational Diabetes Mellitus (GDM) from the start to the end of the gestational period. *For the diagnosis of GDM, glucose, weight, food intake and daily water consumption were quantified to establish clinical signs of Diabetes Mellitus. &From the diagnosis of GDM onwards, glucose, weight and food and water intake were quantified on a weekly basis. +Deaths occurred from day 7 (IP200 and IP230 1 death, SC200 2 deaths), day 8 (IP200 1 death, IP230 2 deaths, SC230 1 death), day 9 (IP200, IP230 and SC230 1 death, SC200 2 deaths) and day 13 (SC200 1 death).

vaginal plug considered to be indicative of pregnancy, and the mice were monitored for the following five days. On the fifth day of mating and confirmation of pregnancy, STZ was administered (Figure 1). Streptozotocin (U-9889) from ChemCruz® (Cat. No. 18883-66-4, Dallas, TX, USA) was administered in a 0.5 M Citrate Buffer solution with a pH of 4.5 (V WR brand; Cat. No. J60024-AP, Ward Hill, MA, USA) intraperitoneally and subcutaneously. Two doses were used for each route of administration: 200 mg/kg and 230 mg/kg[43].

Biochemical and Anthropometric Parameters

Glycaemia

Glycaemia was quantified using a One Touch® Select Plus Flex Glucometer (Cat. No. AW06984804A, Zug, ZG, Switzerland) *via* capillary puncture in the caudal vein. After STZ was administered, postprandial blood glucose was monitored for the next five consecutive days. A diagnosis of DM was confirmed taking into account the criteria established by the American Diabetes Association (ADA) in 2018 (i.e., a sustained glucose concentration ≥ 200 mg/dL, the presence of polyphagia, polydipsia, and changes in body weight)[44].

Insulin and HOMA Index Calculations

The concentration of insulin in the blood before and after inoculation with STZ was quantified to determine functional alterations in insulin secretion. Such alternations could be either due to an absence or deficiency in the conformation of the hormone structure, with the consequent appearance of IR and, therefore, a diagnosis of GDM. To quantify insulin levels, blood samples were collected from the female rats *via* puncture of the retroocular plexus (400 µL) with a heparinized Pasteur pipette; the animals were under general anesthesia, with a mixture of 5 mg/kg of xylazine and 100 mg/kg of ketamine administered intramuscularly[45] at two times: 1) when the gestation of the females was confirmed [the fifth day after mating (Figure 1)], and 2) after the birth of the pups (i.e., between days 23 and 26 of the experiment). Once the sample was taken, the animal was sacrificed *via* cervical dislocation.

The blood samples were centrifuged for 10 minutes at 2,000 revolutions per minute (RPM) to obtain plasma. Insulin concentration was quantified using an Enzyme-Linked Immuno Sorbet Assay (ELISA) test with a commercial ENZO Insulin Kit (Cat. No. EZN-KIT141-000,

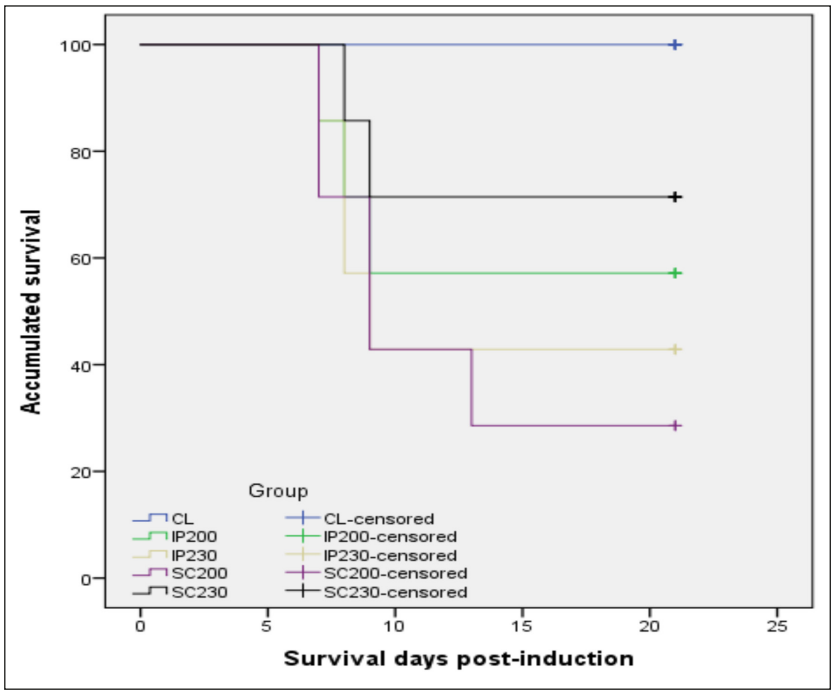

Figure 2. Cumulative survival rate expressed as a percentage (%) of female CD1 mice 21 days after streptozotocin inoculation. The Kaplan-Meier method was used for survival analysis; censored data represent the number of mice alive at the end of follow-up. Comparison between groups was performed with Log Rank (Mantel-Cox) analysis; differences were considered statistically significant with a $p < 0.05$. Control (CL), Intraperitoneal 200 mg/kg (IP200), Intraperitoneal 230 mg/kg (IP230), Subcutaneous 200 mg/kg (SC200), and Subcutaneous 230 mg/kg (SC230). *Number of cases where the event (death) was not recorded at the end of follow-up expressed as a percentage.

Table I. Glucose concentration of female CD1 mice inoculated with streptozotocin.

	CL	IP200	IP230	SC200	SC230	
	Mean ± SD mg/dL (n = 7)	Mean ± SD mg/dL (n = 7)	Mean ± SD mg/dL (n = 7)	Mean ± SD mg/dL (n = 7)	Mean ± SD mg/dL (n = 7)	*p*-value
[+]Day 0	127 ± 9	133 ± 23	124 ± 16	139 ± 36	129 ± 6.7	0.716
[&]Day 5	146 ± 23	130 ± 23	131 ± 20	126 ± 10	119 ± 17	0.152
[**]Day 10	161 ± 25	341 ± 83	340 ± 16	281 ± 15	359 ± 19	0.149
[#]23 at 26 days	122 ± 21	599 ± 0.577	597 ± 4	599 ± 0.707	537 ± 12	0.001[*]

Values represent the Mean ± Standard Deviation (SD) of glucose concentrations in milligrams per deciliter (mg/dL). One-way ANOVA was performed to compare differences between groups; differences were considered statistically significant at $p < 0.05$[*]. Control (CL), Intraperitoneal 200 mg/Kg (IP200), Intraperitoneal 230 mg/Kg (IP230), Subcutaneous 200 mg/Kg (SC200), and Subcutaneous 230 mg/Kg (SC230). Data represent the experiment in duplicate, with the same sample size (n = 7). [+]Day 0, start of the experiment, week 7 of life of the females. [&]Day 5 after mating and prior to inoculation with streptozotocin. [**]Day 10 after inoculation with streptozotocin, with a confirmed diagnosis of GDM. [#]Days 23 to 26 of the experiment, end of gestation, after the birth of the pups.

Farmingdale, NY, USA) following the supplier's recommendations. Next, the Homeostatic Model Assessment of Insulin Resistance (HOMA-IR index) was calculated to determine the degree of IR, using the following formula[46]:

$$\text{HOMA} = \frac{[(\text{Glycaemia (mg/dL)}/18.2)*\text{Insulin (mU/dL)}]}{22.517}$$

Animal Weight

The animals were weighed using an Ohaus™ Triple Beam 700/800 Series mouse scale (Cat. No. 2,729.439, Morris, NJ, USA) prior to STZ administration to establish baseline values. Additional weight measurements were obtained once a week to monitor weight variations.

Food and Water Quantification

To establish the presence of polyphagia and polydipsia, food and water consumption were quantified for five consecutive days from the administration of STZ. The animals' feed was weighed daily using an Ohaus™ CS series Model CS200-001 scale (Cat. No. 72212663, Parsippany, NJ, USA) to calculate feed consumed based on the initial weight of the feed placed on the rack. The volume of water consumed was determined using a 50 mL test tube. In both cases, the calculation was made by subtracting the initial volume of food and water from the amount of food and water that remained at the end of the week.

Statistical Analysis

Body weight, food consumption, water consumption, glucose and insulin levels, and HOMA index variables are reported as mean ± standard deviation (SD). Between-group comparisons were made using one-way analysis of variance (ANOVA) with Tukey's post-hoc test to identify intra-group differences. For the survival analysis, the Kaplan-Meier method was used to determine the survival rate after inoculation. Log-rank analysis (Mantel-Cox) was performed to compare groups. Differences were considered to be statistically significant for *p*-values lower than 0.05. All of the data were analyzed using the statistical software SPSS Version 23 for Windows (IBM Corp., Armonk, NY, USA).

Results

Postprandial Glucose and Survival Rate of Females with GDM and their Offspring

At seven weeks of life, the females exhibited glycemia levels of 130 ± 5 mg/dL. The mean glucose concentration quantified on the fifth day after mating and prior to STZ inoculation was very similar (130 ± 8 mg/dL), and there were no significant differences between groups [F (4, 30) = 0.528, $p = 0.716$ and F (4, 30) = 1.817, $p = 0.152$, respectively]. Glycaemia status changed beginning on the 10th day of the experiment once a diagnosis of GDM was confirmed [F (4, 30) = 1.829, $p = 0.149$] and persisted until the end of pregnancy [F (4, 30) = 111.851, $p < 0.001$], with significantly

Table II. Insulin concentration and HOMA Index calculation of female CD1 mice inoculated with streptozotocin..

	CL	IP200	IP230	SC200	SC230	
	Mean ± SD mg/dL (n = 7)	Mean ± SD mg/dL (n = 7)	Mean ± SD mg/dL (n = 7)	Mean ± SD mg/dL (n = 7)	Mean ± SD mg/dL (n = 7)	*p*-value
&HOMA-IR	0.070 ± 0.007	0.060 ± 0.005	0.074 ± 0.010	0.068 ± 0.003	0.062 ± 0.009	0.013*
#HOMA-IR	0.080 ± 0.016	0.253 ± 0.030	0.246 ± 0.021	0.214 ± 0.038	0.275 ± 0.029	0.001*

Values represent Mean ± Standard Deviation (SD) of insulin concentrations in picograms per deciliter (pg/dL) and HOMA Index. One-way ANOVA was performed to compare differences between groups, differences were considered statistically significant at $p < 0.05$*. Control (CL), Intraperitoneal 200 mg/Kg (IP200), Intraperitoneal 230 mg/Kg (IP230), Subcutaneous 200 mg/Kg (SC200), and Subcutaneous 230 mg/Kg (SC230). Data represent the experiment in duplicate, with the same sample size (n = 7). &Day 5 after mating and prior to inoculation with streptozotocin. #Days 23 to 26 of the experiment, end of gestation, after the birth of the pups.

elevated glucose levels noted at both times in all groups (Table I).

Losses (deaths) were observed beginning on the seventh day after STZ inoculation in the study groups (Figure 1). The group with the largest number of deaths was SC200 (five females deceased), followed by IP230 (four females deceased) and IP200 (three females deceased). The SC230 group exhibited the smallest number of deaths during the experiment (two females deceased; Figure 2). At the end of the gestational period (18-21 days after mating), the highest glucose concentration was observed in the IP200 and SC200 groups [$F(4, 30) = 111.851$, $p < 0.001$], and the SC200 group exhibited the highest death rate (Figure 2). In contrast, survival amongst animals inoculated with STZ was higher in the SC230 group (71%; n = 5), followed by the IP200 group (57%; n = 4; Table I). Both the IP200 and IP230 groups had a death rate of 86% for both groups, and two deaths were noted in the SC200 group. Between the 8th and 10th day, after inoculation, one death per day was recorded in each group (Figures 1 and 2).

Females inoculated with STZ that reached a full gestational term tended to birth a lower number of pups compared with females in the CL group. No pups were birthed in the IP230 or SC200 groups; both groups were characterized by the lowest survival rate (only three and two females survived in each group, respectively). Four females survived in the IP200 group, which was marked by a record of 15 births (an average of 2.1 pups per female). The group with the largest number of pups born was SC230 (57 pups; an average of 8.1 pups per female). In the CL, no female deaths were observed, and 73 pups were birthed (i.e., an average of 10.5 pups per female; Table I).

Quantification of Insulin Concentrations and HOMA Index Calculations

We did not find significant differences in insulin concentrations in the animals prior to STZ inoculation; the mean concentration was 0.7431 ± 0.09 pg/dL. At the end of pregnancy, there were no differences in insulin concentration [$F(4, 30) = 1.065$, $p = 0.391$] between the IP200 (0.7433 ± 0.01 pg/dL) and SC230 (0.7440 ± 0.01 pg/dL) groups. In terms of HOMA-IR were observed significant differences on the fifth day; the IP230 group was the highest [$F(4, 30) = 3.787$, $p = 0.013$), the SC200, SC230, and IP200 groups were found to be below, compared with the Control group (Table II).

At the end of the animals' pregnancies, HOMA-IR increased in all groups ($p < 0.001$), compared with the CL and the initial values at day 5 ($p < 0.001$). The SC230 group had the highest HOMA-IR value, followed by the IP200 group. Furthermore, those two groups exhibited the highest survival rates ($p < 0.001$) (Table II, Figure 2).

Changes in Weight and Food and Water Intake of CD1 Females with and Without DMG

The average weight of the 7-week-old females prior to mating was 22.7 g. The average weight of the animals before inoculation but after mating (i.e., on the fifth day of the experiment) was 26.5 g. Once the females were inoculated with STZ, we observed weight variations among the study groups: on the 10th day of the experiment, females in the SC200 group and the SC230 group lost weight, compared with the CL group [$F(4, 30) = 7.551$, $p < 0.001$).

At the end of the gestation period, we also observed weight variations among the different

groups [F (4, 30) = 22.265, $p < 0.001$). Animals in the SC200 group tended to weigh the least, and animals in the IP200 and SC230 groups had similar mean weights; however, all remained below the mean weight of the Control group (Table III). We attribute the weight differences to the number of pups that the females birthed: as described in the survival section, some females had no live pups. The differences in weight may also be due to variations in food consumption among the groups. After the seventh week after the experiment began, the average food (water) consumption of the animals was 31.28 g per week (49.99 mL per week). That consumption increased progressively in the experimental groups in all cases compared with the CL (Table III).

Discussion

There was increasing interest in using STZ for inducing diabetes in animals because the drug reproduces clinical characteristics similar to those presented by patients with diabetes[47]. Such animal models have shed light on the diabetogenic mechanisms of DM and have enabled researchers to propose new therapeutic strategies focused on minimizing comorbidities associated with the condition[16]. However, before administering the drug, it is necessary to consider important aspects that might interfere with the results of the hyperglycaemic state (e.g., the species used, the age of the animals when they are exposed to the drug, the dosage, and the route of administration). Pharmacologically, the bioavailability of a drug depends on the route of administration[19]. When a drug is given orally, it must pass through the digestive tract, which can result in drug degradation due to the action of digestive enzymes, the acidic pH of the intestine, and hepatic metabolism, thereby reducing the desired effect[48]. Instead, intravenous and intraperitoneal routes reduce the likelihood of degradation, increase the bioavailability of a drug, and amplify its effect with a shorter expo-

Table III. Comparison of weight, food and water consumption of female CD1 mice inoculated with streptozotocin at different times of the experiment.

	CL	IP200	IP230	SC200	SC230	
	Mean ± SD (n = 7)	Mean ± SD (n = 7)	Mean ± SD (n = 7)	Mean ± SD (n = 7)	Mean ± SD (n = 7)	*p*-value
[+]Day 0						
Weight (g)	27.3 ± 0.673	21.6 ± 2.4	24.2 ± 3.2	19.7 ± 2.2	20.6 ± 1.6	< 0.001*
Food (g)	35 ± 0.216	30 ± 0.241	33 ± 0.216	28 ± 0.216	28 ± 0.216	0.001*
Water (mL)	50 ± 0.216	50 ± 0.216	54 ± 0.216	41 ± 0.180	51 ± 0.180	0.001*
[&]Day 5						
Weight (g)	26.4 ± 0.764	25.6 ± 1.3	27.2 ± 1.9	25.1 ± 0.969	24.7 ± 1.3	0.012*
Food (g)	20 ± 0.216	27 ± 0.216	19 ± 0.216	24 ± 0.216	30 ± 0.216	0.001*
Water (mL)	46 ± 0.216	50 ± 0.216	100 ± 0.216	50 ± 0.216	50 ± 0.216	0.001*
[**]Day 10						
Weight (g)	27.4 ± 0.763	25.5 ± 1.7	28 ± 2.4	22.9 ± 1.8	24.7 ± 2.5	< 0.001*
Food (g)	30 ± 0.216	44 ± 0.216	34 ± 0.216	42 ± 0.216	38 ± 0.216	0.001*
Water (mL)	50 ± 0.216	95 ± 0.216	69 ± 0.216	199 ± 0.216	87 ± 0.216	0.001*
[#]23 at 26 days						
Weight (g)	50.1 ± 8.3	28.3 ± 3.2	37.3 ± 10	26.9 ± 1.6	29.5 ± 3.3	< 0.001*
Food (g)	30 ± 0.216	44 ± 0.228	36 ± 0.153	47 ± 0.141	39 ± 0.255	0.001*
Water (mL)	49 ± 0.216	128 ± 0.238	75 ± 0.265	200 ± 0.141	100 ± 0.255	0.001*

The values represent the Mean ± Standard Deviation (SD) of body weight (g), food (g) and water (mL) consumption. One-way ANOVA was performed to compare differences between groups, differences were considered statistically significant at $p < 0.05$*. Control (CL), Intraperitoneal 200 mg/Kg (IP200), Intraperitoneal 230 mg/Kg (IP230), Subcutaneous 200 mg/Kg (SC200), and Subcutaneous 230 mg/Kg (SC230). Data represent the experiment in duplicate, with the same sample size (n = 7). [+]Day 0, start of the experiment, week 7 of life of the females. [&]Day 5 after mating and prior to inoculation with streptozotocin. [**]Day 10 after inoculation with streptozotocin, with a confirmed diagnosis of GDM. [#]Days 23 to 26 of the experiment, end of gestation, after the birth of the pups.

sure time[49]. For example, intraperitoneal inoculation generates greater plasmatic bioavailability in a shorter amount of time compared with subcutaneous inoculation; in contrast, intraperitoneal or subcutaneous drug administration did not change concentrations of the drug measured in the brain[50]. The authors of that study, Mostafavinia et al[51], administered alloxan and STZ in different doses and routes of administration to induce T1DM. The team concluded that the optimal route of administration was subcutaneous, which is consistent with the results obtained in this study[51].

Gestational Diabetes in Female CD1 Mice and Body Weight

The hyperglycemic effect of STZ is dictated *via* the dose and route of administration of the drug[18]. For example, in 6-week-old rats, high-lipid diets were used for 3-4 weeks prior to inoculation with STZ at single doses of 30 mg/kg intraperitoneal; that procedure resulted in a diagnosis of diabetes with a serum glucose level of 300 mg/dL after three days of follow-up[52-54]. In mice, the induction was carried out with inoculation of STZ at 60 mg/kg for five consecutive days intraperitoneally without the use of a high-lipid diet; in this case, a diagnosis of diabetes was established with glucose concentrations ≥ 250 mg/dL[55,56]. In this study, single doses of 200 or 230 mg/kg were administered intraperitoneally or subcutaneously without fasting or a high-lipid diet prior to inoculation; glucose peaks were observed from the first day of inoculation in the SC200 group, and in the remaining groups, hyperglycemia was observed from day 3 onwards with concentrations ≥ 300 mg/dL. Despite the variations in dose, our results partially agree with those that were previously reported[57], particularly in terms of glucose concentrations. Only a few protocols have been established to induce GDM; most of them are focused on T1DM or T2DM[1-3]. The focus of our protocol was to induce GDM in pregnant females using a single dose of STZ and without relying on a high-lipid diet. We also aimed to shorten the length of care of the animals; compared with other protocols in which induction is performed from weaning[28], we were able to maintain the rodents for four fewer weeks. To confirm a diagnosis of GDM, we adopted the criteria established by the ADA: a postprandial glucose concentration ≥ 200 mg/dL and the presence of polyphagia, polydipsia, and weight loss[44].

As noted above, the glucose concentrations of the animals changed beginning on the first day. Our findings also revealed that body weight remained normal until the administration of STZ. From day 10 after inoculation with STZ and until the end of gestation, we observed significant weight decreases in the IP200, SC200, and SC230 groups. This finding is consistent with previous reports[55] that noted weight loss from day 20 of inoculation with 60 mg/kg of STZ. A decrease in body weight is related to protein loss or its degradation; in diabetic rats inoculated with STZ, a loss of up to 8 grams of protein has been reported seven days after inoculation[58]. Structural protein loss or degradation caused by hyperglycemia may explain the weight loss, high mortality, and zero birth rates observed in the SC200 group.

Increased Mortality and Decreased Fertility Related to GDM

Mortality rates of female rats with STZ-induced diabetes may be associated with the route of administration and the dosage of the drug. It has been reported that doses greater than 70 mg/kg can be lethal for some animal models[59,60]. We observed the same to be true: the group of animals inoculated subcutaneously with 200 mg/kg exhibited the highest mortality rate; the next-highest mortality was observed in the group inoculated intraperitoneally with 230 mg/kg. However, the group inoculated subcutaneously with 230 mg/kg had a low rate of mortality, which indicates that the route of drug administration influences mortality more so than the dose itself. In a study of guinea pigs carried out by Podell et al[61], mortality caused by intraperitoneal or subcutaneous inoculation with STZ at a dose of 300 mg/kg was related to acute cytotoxicity in the intestinal epithelium and necrosis in the renal tubules; the results did not change when lower doses of the drug were administered[61]. In that study, the animals also developed mild peritonitis and peritoneal fibrosis. Another side effect of STZ is ovarian alteration and delayed oocyte maturation, which can cause a decrease in fertility[62]. That fact may explain the reduction in the number of births we observed in our study (there were no births in the IP230 and SC200 groups, and there were only 15 births per female in the IP200 group). Another factor related to fertility is the age of an animal when it is exposed to STZ; female mice inoculated at early ages exhibited a smaller number of implantations during gestation and a larger number of embryonic deaths[63]. Hyperglycemia can cause physiological and metabolic changes by affecting glucose transporters (GLUT), which leads to apoptosis

of blastocysts that interrupts cell differentiation, causing malformations and fetal death[64,65].

Presence of Polyphagia and Polydipsia in Female CD1 Mice Inoculated with STZ

The classic signs of diabetes are related to compensatory processes to maintain the homeostasis of an organism[66]. Polyphagia, polydipsia, polyuria, and weight loss are the result of metabolic adaptations to ensure compatibility of life with high blood glucose concentrations[44]; the magnitude of the symptoms is determined by the plasmatic glucose concentration[66]. In the study by Barragán-Bonilla et al[67], female and male Wistar rats were inoculated with two different doses of STZ: 70 mg/kg and 90 mg/kg. The authors observed that animals in the 90 mg/kg group consumed more food compared with the animals who received only 70 mg/kg; these results held regardless of animal sex[67]. In another study[60], male and female rats were inoculated either subcutaneously and intraperitoneally with a single dose of 100 mg/kg at 2 and 5 days of life. Ninety-five percent of the rats died on the fifth day.

On the other hand, rats inoculated subcutaneously on the second day consumed more food than animals inoculated intraperitoneally; the former also exhibited better survival rates. The results of this study agree with those previous literature (i.e., doses above 200 mg/kg led to higher feed consumption; Table III). The same behavior was observed with water consumption. Lo et al[68] reported increased water consumption in mice inoculated with 50 mg/kg of STZ compared with animals in their control group[68]. Another study[69] carried out in male Wistar rats subjected to moderate exercise revealed that sedentary rats induced with 50 mg/kg of STZ consumed more water than rats inoculated with the same dose, but that performed moderate exercise and those that were not inoculated. Our findings support those data: with high plasma glucose concentrations, polydipsia becomes more noticeable.

There are several factors to consider when choosing a diabetes induction model; the most relevant are the species, the route of administration, the dose, potential adverse effects, and the objectives of the study. A subcutaneous route for pharmacological inoculation has advantages over an intraperitoneal route, including easy access, lower error rate, and fewer instances of adverse effects[19]. The selection of the pharmacological dose is also key to the survival and action of the model to be used. When comparing the two routes of administration of STZ, we observed that a subcutaneous route, plus a single high dose of the drug, was the most effective for inducing DM. The results of this study reveal that a dose of 230 mg/kg is quite effective for inducing DM in pregnant female rats. We found that it was possible to reproduce sustained hyperglycemia and classic symptoms such as polyphagia, polydipsia, weight loss, and greater survival and fertility rates. We accordingly conclude that a subcutaneous route at a single dose of 230 mg/kg of STZ is adequate to replicate a model of DM during pregnancy.

Conclusions

The administration of 230 mg/kg of STZ *via* a subcutaneous route was the most effective method for inducing GDM in rats. This route has several advantages compared with previously used models. For example, it reduces physiological stress because animal handling is minimized, and it is often low-cost because repeated administration of the drug is not necessary. Furthermore, a high-lipid diet is not required to potentiate the diabetogenic effect of the drug. In addition, high survival and birth rates were achieved, and the offspring were free of teratogenic effects. We conclude that the subcutaneous administration of STZ at a single dose of 230 mg/kg is adequate to replicate a model of GDM.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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Ethics Approval

The research was approved by the Research Ethics Committee of the Faculty of Medicine of the UAEMéx (CONBIOETICA-15-CEI-002-20210531), endorsed by the National Bioethics Commission (CONBIOÉTICA).

Informed Consent

Since the study was carried out in an animal model, informed consent is not required. However, the standards and guidelines established in the Official Mexican Standard NOM-062-ZOO-199, "Technical specifications for the production, care and use of laboratory animals" as well as the ARRIVE standards (Animal Research: Reporting of In Vivo Experiments) for animal studies were followed.

Data Availability

The datasets generated during the current study are available from the corresponding author upon reasonable request.

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Authors' Contributions

RGCA: experimental work, implementation of the animal model and writing of the manuscript. MCBE: conception and design of the study, analysis of samples, writing of the manuscript and statistical analysis. RAAA and AMIM: analysis of biological samples and drafting the article. GLAL: statistical analysis, interpretation of data and manuscript review. ARE: maintenance and obtaining biological samples. All the authors supervised, validated and approval the final version of the article to be published.

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