Characterization of Streptozotocin-induced Diabetic Rats and Pharmacodynamics of Insulin Formulations

Jae-Jeong Lee, Ho-Young Yi, Jae-Won Yang, Jun-Seop Shin, Jai-Hyun Kwon, and Chan-Wha Kim†

Graduate School of Life Sciences and Biotechnology, Korea University, Seoul 136-701, Korea

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Morphological and functional changes of rat pancreatic islets caused by administration of streptozotocin (STZ) and the bioavailability of insulin formulations administered to STZ-induced diabetic rats with fasting (12 h) or non-fasting were investigated. Islets isolated from normal rats maintained a good three-dimensional structure and the islet yield was 962.5 ± 86.5 islet equivalent number (IEQ, islets converted to an average diameter of 150 µm). In the diabetic group (≥500 mg/dl blood glucose), the islet yield was only 44.4 ± 8.3 IEQ and the islet was severely damaged. The minimum reduction of blood glucose of each formulation, such as insulin solution, microcrystal, and insulin microcrystal capsule, was shown to be 11.3, 11.0, and 16.3 mg/dl, respectively, at 6 h in fasting with diabetic rats. These results indicated that the administration of insulin formulations to the fasting groups increased the severe hypoglycemic effect of insulin action more than in non-fasting diabetic rats. The diabetic rat with fasting has a regulatory disorder in maintaining the blood glucose level. Accordingly, the validity of pharmacological availability as an optimal modeling of insulin formulations is best in non-fasting STZ-induced diabetic rats.

Key words: insulin; streptozotocin; diabetes; pharmacodynamics; islet

Diabetes mellitus (DM) is classified into two types.1,2) Type 1 diabetes, also known as insulin-dependent diabetes mellitus (IDDM), is characterized by the autoimmune destruction of pancreatic β-cells.4,5) Type II diabetes, also known as non-insulin-dependent diabetes mellitus (NIDDM), is a complex disease characterized by target organs, such as liver, muscle, and adipocytes.7,8)

Streptozotocin (STZ)-induced diabetic animals have been used for a model of IDDM. In general, STZ given at a single dose of over 50 mg/kg causes massive β-cell destruction and permanent hyperglycemia in various experimental animals.10-12)

In this study, morphological and functional changes of rat pancreatic islets caused by administration of STZ were investigated both in vivo and in vitro. In order to assess the direct effect of STZ on the rat pancreatic islets, the islet number, expressed as 150 µm equivalent size (IEQ),13,14) islet morphology with dithizone staining,15) blood glucose (BG) levels, and intraperitoneal glucose tolerance test (IPGTT)16) were examined.

The administration of insulin for the treatment of IDDM and some cases of NIDDM usually controls the BG level sufficiently to maintain a normal physiological state.17) One of the animal experimental models to study the administration of insulin formulations, such as insulin solution, insulin microcrystals and insulin microcrystal capsules, is the Sprague-Dawley rat with diabetes induced by STZ.18) In order to measure pharmacokinetic and pharmacodynamic properties of insulin formulations,19-21) we used STZ-induced diabetic rats with fasting or non-fasting.

Materials and Methods

Materials. Insulin powder of bovine origin, STZ, collagenase, Ficoll, and dithizone were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco’s Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco-BRL (Grand Island, NY, USA). Seven-week-old male Sprague-Dawley (SD) rats (Dae-Han Experimental Animal, Seoul, Korea) were housed in an isolator cage system in air-conditioned animal chamber at 22 ± 1°C.

Diabetes induction. Male SD rats were given no food for 3 h before diabetes was induced with STZ. They received a single intraperitoneal (IP) injection of 60 mg/kg STZ freshly dissolved in 0.05 M chilled sodium citrate buffer, pH 4.5. Normal rats were injected with the equivalent volume of citrate buffer. STZ-induced diabetic animals with diabetic status (≥300 mg/dl) for 2 weeks were taken for the study.

† To whom correspondence should be addressed. Tel: +82-2-3290-3439; Fax: +82-2-3290-3957; E-mail: cwkim@korea.ac.kr
Blood glucose concentration measurement. Blood samples were collected from the rat tail vein. The blood glucose (BG) levels of the rats were monitored daily by using an One-touch blood glucose monitoring system (LifeScan Inc., Milpitas, CA, USA), and rats were considered to be diabetics when three consecutive BG values were above 250 mg/dl.

Intraperitoneal glucose tolerance test. Intraperitoneal glucose tolerance tests (IPGTT) in the rat administered 60 mg/kg STZ and in normal rat were done on rats with a BG level of normal (80–120 mg/dl), 100–150, 200–300, 300–350, and > 500 mg/dl after STZ administration. These animals were not fed for 12 h before the test and then intraperitoneally administered a glucose load of 2 g/kg body weight.

Pancreatic islet isolation. SD rats were killed by cervical dislocation. Briefly, the pancreas was exposed and distended by the intraductal injection of collagenase (0.7 mg/ml, Type XI, Sigma Chem. Co.). The distended pancreas was then immediately dissected out and incubated at 37°C for 20 min. The reaction was stopped by adding ice-cold DMEM containing 10% FBS, and the homogenate was washed twice with DMEM. The islets were purified by discontinuous density gradient separation with Ficoll solution (1.108, 1.096, and 1.037 g/cm³). The isolated pancreatic islets were finally hand-picked with a Pasteur pipette under the stereomicroscope.

Dithizone staining. Dithizone (DTZ) selectively stains β-cells in pancreatic islets. DTZ solution was prepared by dissolving 10 mg of DTZ in 3 ml of absolute ethanol and 60 μl of ammonium hydroxide. Islets were stained by adding 20 μl of DTZ solution to islets in 1 ml of DMEM culture medium.

Assessment of islet yield. Immediately after islet isolation, total islet yield evaluation was done in triplicate using DTZ staining by a newly modified simple counting system for isolated islets. Islets < 50 μm in diameter were excluded from the islet yield. The total volume of islets is expressed in the number of islet equivalents (IEQs)—defined as islets of 150 μm equivalent size in diameter.

Intraperitoneal injection of insulin and pharmacodynamics analysis. Fasting or non-fasting diabetic rats were subdivided into 4 groups. In non-fasting diabetic groups, they received IP injections, whereas in fasting groups, after a 12 h fast, they received IP injections. One of the following drugs was administered by the IP injection:

- Group 1: control, fasting/non-fasting rats who received PBS (pH 7.4).
- Group 2: insulin solution, fasting/non-fasting rats who received the insulin dissolved in PBS (pH 7.4).
- Group 3: insulin microcrystals, fasting/non-fasting rats who received insulin micro-crystals suspended in PBS (pH 7.4). Insulin microcrystals were produced by insulin powder being dissolved in an acetate solution of pH 2.0 and the acidity of the insulin solution was varied using 1 N and 10 N NaOH. Then, when the pH was further raised to 6.0, more than 65% of the insulin crystals produced had a diameter of 5 μm or less.
- Group 4: insulin microcrystal capsules, fasting/non-fasting rats who received the suspension of microcrystals encapsulated with a copolymer of lactic and glycolic acid (PLGA, 50:50) in PBS (pH 7.4). Insulin microcrystals were encapsulated within PLGA microspheres by the multiple emulsion-solvent evaporation technique. In this study, the pharmacodynamic properties of insulin formulations were evaluated by fasting or non-fasting STZ-induced diabetic rats.

Results and Discussion

Changes of the blood glucose concentration and body weight after STZ administration

The metabolic characterization was based on an IPGTT, blood glucose (BG), and body weight. The BG level of the normal group was 100 ± 10 mg/dl throughout the observation period of 1–12 days. SD rats with STZ-induced diabetes have reduced body weight, hyperglycemia, and hypoinsulinemia because of damaged insulin-secreting cells in the pancreatic islets. When 50, 60, and 70 mg/kg STZ were administered, then one day after STZ injection of these rats, there was a marked increase of BG levels (from about 300 to 500 mg/dl) (Fig. 1A). One of the rats treated with 70 mg/kg STZ died from hyperglycemia on day 3 and showed severe weight loss (Fig. 1B). IP administration of the STZ at 60 and 70 mg/kg body weight significantly reduced the body weight in STZ-diabetic rats compared to the normal, but body weight increased in 50 mg/kg diabetic rats (Fig. 1B). Twelve days after STZ administration, the STZ untreated normal group gained 45 g body weight progressively and the group treated with 50 mg/kg STZ also gained 23 g body weight in 12 days. However, the increase in the body weight of rats treated with 50 mg/kg STZ was much lower than that of the normal rats. Whereas, the groups treated with 60 and 70 mg/kg STZ lost the body weight continuously for 12 days (22 g and 55 g, respectively). Therefore, the treatment of 70 mg/kg STZ was not appropriate for this study. At 50 mg/kg, induction of diabetes was difficult and not reproducible, and the rate of inducing diabetes was about 60%. Based on these results, 60 mg/kg STZ was selected as an optimum dose to induce diabetes.
Changes of BG values were measured after STZ treatment of SD rats at different doses for 12 days. The changes of BG concentration when the SD rats are administered 50 (\(\bigcirc\), \(n = 3\)), 60 (\(\bigtriangledown\), \(n = 4\)), and 70 mg/kg STZ (\(\bigtriangledown\), \(n = 3\)) and the control (PBS, \(\bullet\), \(n = 6\)). Each plot denotes the mean ± SEM.

Changes of body weight in SD rats after a single i.p. injection of various doses of STZ. \(\bullet\), \(\bigcirc\), \(\bigtriangledown\) and \(\bigtriangledown\) represent for normal (\(n = 6\)), 50 (\(n = 3\)), 60 (\(n = 4\)) and 70 mg/kg STZ (\(n = 3\)). Each plot denotes the mean ± SEM.

Effects of STZ on islet morphology and yield

We investigated the effects of STZ on the functional and histological characteristics of endogenous pancreatic islets in normal and STZ-induced diabetic rats. After two weeks of STZ treatment, the STZ-induced diabetic rat pancreatic islets were stained with DTZ and their morphologies were observed under the phase-contrast microscope. The diabetic rats had severe diabetes as represented by decreased body weight, high basal BG level, and severely reduced total islet mass (with a mass representing 73.0 ± 9.7 % ± 4.6 ± 9.6%) of the related normal islet mass. The morphology of normal rat pancreatic islets was round, compact, and well stained with DTZ (Fig. 2A). While diabetic pancreatic islets were collapsed, amorphous, and dimly stained with DTZ at the BG level of above 200 mg/dl (Fig. 2C, D and E), islets which were isolated from rats with BG level of 100 ± 10 mg/dl were compact and maintained a round shape (Fig. 2B). However, there was a remarkable reduction in the number of islets as the BG level increased to 100 ~ 150, 200 ~ 300, 300 ~ 350, and ≥ 500 mg/dl (Fig. 3). The islet yield was 962.5 ± 86.5 IEQ/pancreas in the normal group, 694.0 ± 94.9 IEQ/pancreas in the diabetes group with a BG level 100 (ranging from 100 to 150 mg/dl), 490.10 ± 148.82 IEQ/pancreas in the diabetic group with a BG level 200 (ranging from 200 to 300 mg/dl), 123.8 ± 15.2 IEQ/pancreas in the diabetes group with a BG level
300 (ranging from 300 to 350 mg/dl) and 44.5 ± 8.3 IEQ/pancreas in the diabetes group with a BG level higher than 300 mg/dl (n = 3, each). These results indicated that the administration of increased STZ to animals decreases the recovery of viable islets, and reduces beta-cell function.

**Intraperitoneal glucose tolerance test**

We did IPGTT at various BG concentrations after STZ administration to further clarify the characteristics of the STZ-induced diabetic rat model. The hypoglycemic property of each group was assessed by IPGTT at a dose of 2 g/kg (body weight) glucose in normal and STZ-induced diabetic rats (Fig. 4). The rats were selected according to BG levels and given no food for 12 h before the administration of glucose. The initial BG levels in diabetic rats after fasting for 12 h were different from the non-fasting BG level of these rats. The fasting condition contributed to the decline in BG level. BG levels in each case were measured at various times. In normal control rats, the BG level increased moderately to 140 ± 10 mg/dl after glucose infusion, but it was rapidly restored to normal within 2 h (Fig. 4). There was no significant difference in BG levels between the normal and diabetic groups (100 and 300 mg/dl), and the normalized BG levels maintained for at least 2 h after the glucose infusion. In the diabetic groups with BG levels higher than 300 and >500 mg/dl, the glucose level increased above 400 mg/dl, and declined slowly. In diabetic groups with BG level of 100–300 mg/dl, the BG level increased about 250 mg/dl, which was restored to normal within 2 h. Normal, BG 100–150 mg/dl, BG 200–300 mg/dl level (2 h), produced a significant BG-lowering effect in the diabetic rats when compared to the BG 300–350 mg/dl and BG >500 mg/dl (<8.5 h). This results indicated that glucose-stimulated insulin secretion is markedly impaired in the BG level of >300 mg/dl. Thus, we examined the effects of insulin formulations by in BG 300–350 mg/dl STZ-induced diabetic model.

**Insulin administration and pharmacodynamic analysis**

To evaluate the effects of insulin on the BG level, the STZ-induced diabetic rats with fasting or non-fasting were IP injected with different insulin formulations. In order to arrest insulin releasing, fasting groups were without food for 12 h before insulin administration by IP injection. Changes of BG levels after administration of various insulin formulations are shown in Fig. 5A and 5B. Fasting groups are shown in Fig. 5A, when insulin solution and microcrystals were administered, a significant decrease of glucose level was seen, and then, their BG levels were shown in a steady-state for 15 h. The lowest BG level of the group received microcrystal capsules was 6 h (16.3 ± 8.4 mg/dl), and their BG level gradually recovered and increased. Even though in the control group, their BG level declined remarkably to 57.7 ± 8.8 mg/dl (Table 1). This result was compatible with that of other studies, on the effects of fasting and insulin-induced hypoglycemia in newborn piglets.10) Diabetic rats, fasted for 12 h before insulin administration, underwent hypoglycemic (<60 mg/dl BG) shock in our previous experiments. Shown in Fig. 5B, in case of diabetic rats without fasting, in those groups received insulin solution and microcrystals, their minimum reduction level of BG was similar, as 382.3 ± 15.9 and 394.7 ± 62.3 mg/dl. In diabetic rats, administration of microcrystal capsules decreased their BG level to 75.0 ± 8.2 mg/dl in 2 h (Fig. 5B and Table 1). These results indicated
Fig. 5(A). Changes of the BG Concentration in STZ-Induced Diabetic Rats with Fasting by Insulin Formulations.

The changes of BG concentration when the STZ-induced diabetic SD rats are administered a solution (○, 2 IU, n = 3), microcrystals (▲, 5 IU, n = 3), microcrystal capsules (γ, 20 IU, n = 3), and a control (●, PBS, n = 3). Each plot denotes the mean ± SEM.

Fig. 5(B). Changes of the BG Concentration in STZ-Induced Diabetic Rat with Non-fasting by Insulin Formulations.

The changes of BG concentration when the STZ-induced diabetic SD rats are administered a solution (○, 2 IU, n = 3), microcrystals (▲, 5 IU, n = 3), microcrystal capsules (γ, 20 IU, n = 4), and a control (●, PBS, n = 4). Each plot denotes the mean ± SEM.

Table 1. Pharmacodynamic Analysis of Insulin IP Administration by Using STZ-Induced Diabetic Rats with Fasting (F) and Non-fasting (NF)

<table>
<thead>
<tr>
<th>Insulin Formulations</th>
<th>Dose (IU/kg)</th>
<th>MRBG1 (mg/dl)</th>
<th>TMBG2 (h)</th>
<th>% TRBG3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>F</td>
<td>NF</td>
<td>F</td>
</tr>
<tr>
<td>PBS5</td>
<td>0</td>
<td>57.7±8.8</td>
<td>407.5±27.5</td>
<td>14</td>
</tr>
<tr>
<td>Solution</td>
<td>2</td>
<td>11.3±1.8</td>
<td>382.3±15.9</td>
<td>6</td>
</tr>
<tr>
<td>Microcrystal</td>
<td>5</td>
<td>11.0</td>
<td>394.7±62.3</td>
<td>10</td>
</tr>
<tr>
<td>Microcrystal capsule</td>
<td>20</td>
<td>16.3±8.4</td>
<td>75.0±6.2</td>
<td>6</td>
</tr>
</tbody>
</table>

MRBG1: the minimum reduction of blood glucose.
TMBG2: time at minimum blood glucose.
% TRBG3: the percent of total reduction in blood glucose.
PBS5: phosphate-buffered saline.

that the administration of insulin formulations to fasting groups increased the synergistic effect of insulin on hypoglycemia action than non-fasting groups. The pharmacodynamic properties of insulin formulations were evaluated. Table 1 presents the opportunity to compare the pharmacodynamics of fasting or non-fasting with STZ-induced diabetic rats. The MRBG of insulin solution, crystals and microcrystal capsules was 11.3±1.8, 11.0 and 16.3±8.4 mg/dl, respectively in fasting groups. This result might be due to the fact that the diabetic rats had been fasted.25) Influx of exogenous and endogenous insulin results in a synergistic effect on the BG level decrease.

The %TRBG of the non-fasting group was significantly different from the fasting group. The % TRBG of the non-fasting was shown in insulin formulations, such as insulin solution, microcrystals, and insulin microcrystal capsules (7.5, 18.8, and 30.6%, respectively). Whereas, fasting groups were 88.4, 89.9, and 80.2%, respectively. These results indicated that diabetic rats with fasting have a regulatory disorder in maintaining BG levels. Therefore, in order to investigate the pharmacodynamics of BG level by exogenous insulin injection, an experiment was done on diabetic rats without fasting.

In summary, this study indicates that the diabetic rat model induced by a single IP injection of 60 mg/kg STZ is IDDM, which is characterized by the impaired insulin response to glucose stimulation (IPGTT), islet yield, change of BG concentration, and gain or loss of body weight. In addition, this diabetic rat with fasting or non-fasting may be useful to clarify the mechanism for IDDM in animal experiments and to screen insulin formulations.

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