Streptozotocin-Induced Diabetic Models in Mice and Rats

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Streptozotocin-Induced Diabetic Models in Mice and Rats

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Streptozotocin (STZ) is an antibiotic that produces pancreatic islet β-cell destruction and is widely used experimentally to produce a model of type 1 diabetes mellitus (T1DM). Detailed in this unit are protocols for producing STZ-induced insulin deficiency and hyperglycemia in mice and rats. Also described are protocols for creating animal models for type 2 diabetes using STZ. These animals are employed for assessing the pathological consequences of diabetes and for screening potential therapies for the treatment of this condition.

Keywords: streptozotocin • type 1 diabetes mellitus • hyperglycemia • insulin deficiency • insulin resistance • insulitis • mouse or rat • nicotinamide • high-fat • type 2 diabetes mellitus

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INTRODUCTION

Streptozotocin (STZ) was initially isolated from *Streptomyces achromogenes* in 1960, with its diabetogenic properties not described until 1963 (Rakieten et al., 1963). This action was characterized by Junod et al. (1969) based on earlier work (Junod et al., 1967) showing that the diabetogenic effects are due to selective destruction of pancreatic islet β-cells. As a result of this action, the animals experience insulin deficiency, hyperglycemia, polydipsia, and polyuria, all of which are characteristic of human type 1 diabetes mellitus (T1DM; Kolb, 1987).

Several animal species, including the mouse, rat, and monkey, are sensitive to the pancreatic β-cell cytotoxic effects of STZ, with the rabbit being less so (Lazar et al., 1968). Currently, STZ is most often used to induce diabetes in rats and mice.

Described in this unit are two protocols used to produce STZ-induced diabetes in mice (Basic Protocol 1) and rats (Basic Protocol 2). Basic Protocol 1 employs multiple administrations of low-dose STZ to produce diabetic mice, and is increasingly being used as an animal model for diabetes. Its growing popularity is due to the fact that the resultant pathology resembles human T1DM with chronic pancreatic islet inflammation, insulitis, and insulin deficiency; its lower cost compared to other animal models is also an attaction. The procedure described in Basic Protocol 2 is used to induce diabetes in rats with STZ. While one of the earliest animal models of this condition, it remains popular for this purpose.

While these models are valuable for evaluating treatments for T1DM, the majority of diabetic patients suffer from type 2 diabetes (T2D). Two potentially useful STZ models

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of T2D are being developed, both of which are included in this unit. The first (Basic Protocol 3) entails concurrent administration of nicotinamide to partially protect the β-cells against STZ (Masiello et al., 1998). This model is based on the work of Junod et al. (1969), who investigated the earlier finding of Schein et al. (1967) that nicotinamide protects against the diabetogenic effect of STZ. This compound combination produces a model of insulin-deficient, but not insulin-resistant, T2D. It is characterized by stable, moderate hyperglycemia associated with an approximately 60% loss of β-cell function (Masiello et al., 1998; Ghasemi et al., 2014). However, as most patients with T2D display insulin resistance in addition to impaired insulin secretion, another model (Basic Protocol 4) has been developed to more accurately mimic the human condition. In this case, the animals are exposed to a high-fat diet to produce insulin resistance, followed by administration of a moderate dose of STZ to reduce β-cell capacity (Reed et al., 2000). The result is hyperglycemia, associated with hyperinsulinemia and insulin resistance.

NOTE: All experimental protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must comply with the guidelines as established by the IACUC regarding the care and use of laboratory animals in scientific experiments. They must also comply with governmental legislation, such as the UK Animals (Scientific Procedures) Act, 1986, as amended in 2012 (*https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/308593/ConsolidatedASPA1Jan2013.pdf*).

BASIC PROTOCOL 1

INDUCTION OF TYPE 1 DIABETES MELLITUS IN MICE USING REPEATED LOW DOSES OF STREPTOZOTOCIN

Streptozotocin (STZ) is a highly selective pancreatic islet β-cell-cytotoxic agent that in the past was administered at a single high dose to cause, within 48 hr, complete β-cell necrosis and diabetes (Kolb, 1987; also see Alternate Protocol). However, after administering multiple, low doses of STZ to mice for 5 days, Like and Rossini (1976) noted a delayed onset of hyperglycemia which, for kinetic reasons, could not be due to a direct, rapid, toxic action of the drug. This multiple, low-dose STZ approach only partially damages pancreatic islets, triggering an inflammatory process that causes the further loss of β-cell activity that ultimately results in insulin deficiency and hyperglycemia. This response more closely resembled T1DM in pathogenesis and morphologic changes than the single-high-dose-of-STZ protocol (Like and Rossini, 1976; Kolb, 1987; Kolb-Bachofen et al., 1988; Weide and Lacy, 1991). The multiple, low-dose STZ approach is now widely used to produce an animal model of T1DM (Wu and Huan, 2007).

Using this protocol, diabetes is induced by the administration of multiple, low doses of STZ (40 mg/kg, intraperitoneally; i.p.) to mice on 5 consecutive days. The model is used for testing the effectiveness of potential antidiabetic agents. Screening assays can entail administration of the test agent prior to and/or following induction of diabetes, depending on study objectives.

Materials

C57BL/6 or CD-1 male mice: ~25 g, 8 to 12 weeks old (Jackson Laboratory or Taconic); 12 to 20 animals per treatment group are recommended; Balb/cJ mice are resistant to the induction of diabetes using this treatment regimen (see Zunino et al., 1994).

Standard rodent chow diet (Harlan)

50 mM sodium citrate buffer (enzyme grade; Fisher), pH 4.5: prepared immediately before use

Streptozotocin (STZ; Sigma)

10% (w/v) sucrose (Sigma): prepared just before use

Test compound(s)

Rodent cages
Temperature-, humidity-, and light-controlled housing
1.5-ml microcentrifuge tubes
Aluminum foil
1-ml syringes
25-G needles
One Touch Basic blood glucose monitoring system (Lifescan)

Additional reagents and equipment for injection of mice (Donovan and Brown, 2006a) and blood collection from mice (Donovan and Brown, 2006b)

Prepare animals

1. At least 5 days prior to initiating the experiment, house two to five male mice per cage at 24°C ± 1°C and 55% ± 5% humidity, with a 12-hr light-dark cycle (light on at 8:00 and off at 20:00). Allow the mice free access to food and water.

   Because female mice are less sensitive to this islet-cell toxin, most STZ-induced diabetic mouse studies are conducted on male animals (Kolb, 1987).

   While the protocol detailed below is designed to minimize variability, group sizes of 12 to 20 are recommended given the morbidity associated with the STZ treatment.

2. Weigh all mice accurately to 1 g and randomly divide them into control and experimental groups.

   The number of mice should be equal for each group.

3. On experimental day 1, 4 hr prior to STZ treatment, remove all food from cages for all groups. Provide water as normal.

Treat animals with STZ

4. Weigh 4 mg of STZ into a 1.5 ml microcentrifuge tube and cover the tubes with aluminium foil; use one tube for three mice. Prepare the citrate buffer.

5. Immediately prior to injection, dissolve the STZ in the 50 mM sodium citrate buffer (pH 4.5) to a final concentration of 4 mg/ml.

   Because STZ degrades within 15 to 20 min after dissolving in the citrate buffer, the STZ solution should be prepared immediately before use and injected within 5 min of dissolution.

6. Using 1-ml syringes and 25-G needles, inject the STZ solution i.p. at 40 mg/kg (1.0 ml/100 g) in the experimental group animals. Inject an equal volume of citrate buffer (pH 4.5) i.p. (Donovan and Brown, 2006a) into the control group mice.

   The responses to intravenous (i.v.) and i.p. injections of STZ are equivalent (Like and Rossini, 1976).

7. Return the mice to their home cages. Provide free access to normal food and 10% sucrose water.

8. Repeat steps 3 to 7 on days 2 to 5 (the next 4 consecutive days).

9. On experimental day 6, replace the 10% sucrose water with regular water.

10a. *For studies involving early-stage T1DM:* On experimental day 14 (9 days after the last STZ injection), fast all mice for 6 hr (e.g., from 7:00- 13:00). Analyze blood glucose from a tail-vein blood sample (Donovan and Brown, 2006b) using a One Touch Basic blood glucose monitoring system to ensure hyperglycemia in the STZ-treated subjects.

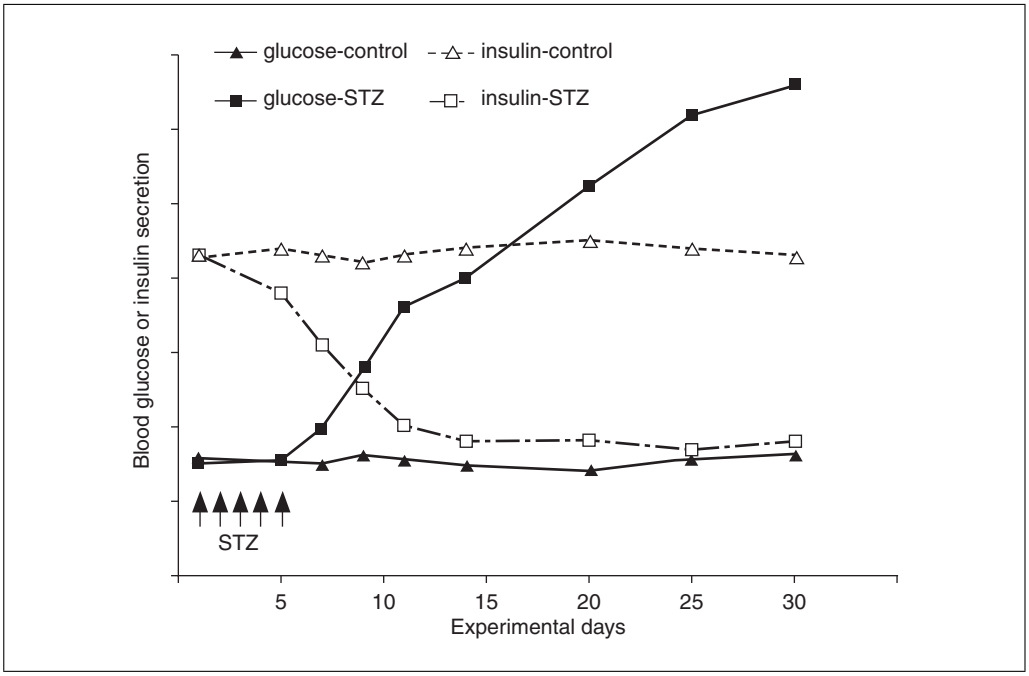

Figure 5.47.1 Schematic representation of the time course of multiple, low-dose STZ-induced diabetes in mice. Mice were treated with STZ (40 mg/kg) or without STZ (vehicle control) for 5 consecutive days. Typical changes in insulin secretion and blood glucose levels are illustrated.

If the diabetic animals are for studying early-stage mechanisms of T1DM or for screening compounds for early treatment of diabetes, the animals are considered suitable for study when the blood glucose levels of the STZ-injected mice is >150 mg/dl (8.3 mmol/liter) and/or statistically higher than in the control mice. Step 10b can be omitted if STZ- injected mice meet these criteria prior to experimental day 28.

If <40% of mice in the STZ-injected group attain a diabetic state by day 14, re-test blood glucose levels as in step 10b.

As blood glucose levels are considered an accurate diagnostic tool for diabetes, there is generally no need to measure blood insulin levels.

10b. *For re-test of animals failing the first test for diabetes and for studies involving later-stage T1DM:* On experimental day 28, fast all mice for 6 hr (e.g., from 7:00-13:00). Quantify blood glucose from a tail-vein blood sample (Donovan and Brown, 2006b) using a One Touch Basic blood glucose monitoring system to confirm STZ injection-induced hyperglycemia.

Fasting glucose levels for mild hyperglycemia should be >150 mg/dl (8.3 mmol/liter) and/or be significantly higher in the STZ-injected mice as compared to the control mice.

Severe diabetes usually develops in ~50% of mice ~3 weeks after STZ injection, with blood glucose levels typically in the >300 to 600 mg/dl (16.7 to 33.3 mmol/liter) range.

If >60% of STZ-injected mice still fail to exhibit mild hyperglycemia by week 4, a second round of STZ injection should be initiated at week 7 by repeating steps 3 to 8, plus step 10a. For the second round of STZ, there is no need to provide the animals with 10% sucrose water, as the incidence of fatal hypoglycemia is much lower than with the first exposure.

A blood glucose level of 18 mg/dl = 1 mM (Hartnell et al., 1990).

11a. *To examine a test agent for its ability to correct diabetes or affect hyperglycemia:* Begin treatment with a test hypoglycemic agent once the diabetic state is established as defined in step 10. Include groups that receive appropriate vehicle injections as

a control. Maintain the duration of treatment with test substance according to the experimental design.

11b. *To study a chronic condition or diabetic complications:* Repeat, at week 7, steps 3 to 8, omitting the 10% sucrose water, to maintain hyperglycemia in the STZ-treatment group (Kunjathoor et al., 1996).

Shown in Figure 5.47.1 are typical insulin secretion and blood glucose levels following administration of multiple, low doses of STZ.

INDUCTION OF TYPE 1 DIABETES MELLITUS IN MICE USING A SINGLE, HIGH DOSE OF STREPTOZOTOCIN

ALTERNATE PROTOCOL

A single, high dose of STZ (200 mg/kg) is directly toxic to pancreatic β-cells, rapidly causing diabetes, with blood glucose levels of >500 mg/dl within 48 hr (Like and Rossini, 1976). Although multiple, low doses of STZ are associated with fewer toxic effects than a single, high dose of STZ, many investigators still prefer the single high-dose STZ approach for generating diabetic animals.

For materials, see Basic Protocol 1.

Prepare animals

1. At least 5 days prior to the initiating the experiment, house two to five male mice per cage at 24°C ± 1°C and 55% ± 5% humidity, with a 12-hr light-dark cycle (light on at 8:00 and off at 20:00) with free access to food and water.

   Because female mice are less sensitive to this islet-cell toxin, most STZ-induced diabetic mouse studies are conducted on male animals (Kolb, 1987).

   While the protocol detailed below is designed to minimize variability, group sizes of 12 to 20 are recommended given the morbidity associated with the STZ treatment.

2. Weigh all mice accurately to 1 g and randomly divide them into control and experimental groups.

   The number of mice should be equal for each group.

3. On experimental day 1, 4 hr prior to STZ treatment, remove food from all animal cages. Provide water as normal.

Treat animals with STZ

4. Immediately prior to injection, dissolve the STZ in sodium citrate buffer (pH 4.5) to a final concentration of 20 mg/ml.

   The STZ solution should be prepared fresh immediately before injection and injected within 5 min of being dissolved. Note the higher dose used for this method as compared to Basic Protocol 1.

5. Inject STZ i.p. (Donovan and Brown, 2006a) into the experimental animals at 200 mg/kg (1.0 ml/100 g). Inject an equal volume of citrate buffer (pH 4.5) i.p. into the control group mice.

6. Return the mice to their cages. Provide normal food and 10% sucrose water and closely monitor the mice every 2 hr for 12 hr for marked hypoactivity, unresponsiveness, or convulsions.

   Some mice will die soon (within 24 hr) after receiving a high dose of STZ due to the rapid and massive β-cell necrosis that results in the release of large quantities of insulin, causing fatal hypoglycemia. If the number of early deaths is >20%, inject the remaining mice i.p. within 6 hr of the STZ treatment with 1 ml of 5% glucose solution instead of

providing 10% sucrose water for drinking, to prevent fatal hypoglycemia (Huang and Wu, 2005).

7. On experimental day 3, replace the 10% sucrose water with regular water.

8a. *For studies involving early-stage T1DM:* On experimental day 10, fast all mice for 6 hr (e.g., from 7 a.m. to 1 p.m.), then measure blood glucose in a tail-vein blood sample (Donovan and Brown, 2006b) using a One Touch Basic blood glucose monitoring system to ensure hyperglycemia.

If the diabetic animals are to be used for assessing early-stage mechanisms of T1DM or for screening drug candidates for treating diabetes, the animals can be employed once hyperglycemia is established or the blood glucose levels are statistically higher in the STZ-treated subjects than in controls. In this case, skip step 8b. Skip step 8a if the diabetic animals are ***not*** *for early-stage assessment of T1DM mechanisms or for screening drug candidates as early treatments for this condition.*

If <40% of the STZ-injected mice become diabetic, retest blood glucose levels as described in step 8b.

As blood glucose levels are considered an accurate diagnostic tool for diabetes, there is generally no need to measure blood insulin levels.

8b. *For re-test of groups failing the first test for diabetes or for studies involving later-stage T1DM:* On experimental day 21, fast all mice for 6 hr (e.g., from 7 a.m. to 1 p.m.). Test the blood glucose level in a tail-vein blood sample (Donovan and Brown, 2006b) using a One Touch Basic blood glucose monitoring system to confirm that the STZ treatment induced hyperglycemia.

Fasting glucose levels for mild hyperglycemia should be >150 mg/dl (8.3 mmol/liter) and/or be significantly higher in the STZ-injected mice than in the control subjects.

Usually at week 3, most STZ-injected mice develop severe diabetes with blood glucose levels in the range of >300 to 600 mg/dl (16.7 to 33.3 mmol/liter).

If >60% of the STZ-injected mice still fail to exhibit mild hyperglycemia, determine whether there are problems with the experimental procedure (see Critical Parameters and Troubleshooting), or use the multiple low-dose STZ approach (see Basic Protocol 1).

A blood glucose level of 18 mg/dl = 1 mM (Hartnell et al., 1990).

9a. *To examine a test agent for its ability to correct diabetes or affect hyperglycemia:* Once the diabetic state is confirmed as specified in step 8, treat the animals with a test anti-hyperglycemic agent. Include as controls animals that receive appropriate vehicle injections.

The duration of the injection and screening period for any given test agents will depend on the question being examined.

9b. *To study a chronic condition or diabetic complications:* Extend the protocol longer, depending on the experimental needs.

The length of the experiment depends on the aims of the investigation, e.g., from several days for acute studies to several weeks for studies on diabetic complications.

Shown in Figure 5.47.2 are some characteristics of a typical mouse diabetic state 3 weeks after injection of a single, high dose of STZ.

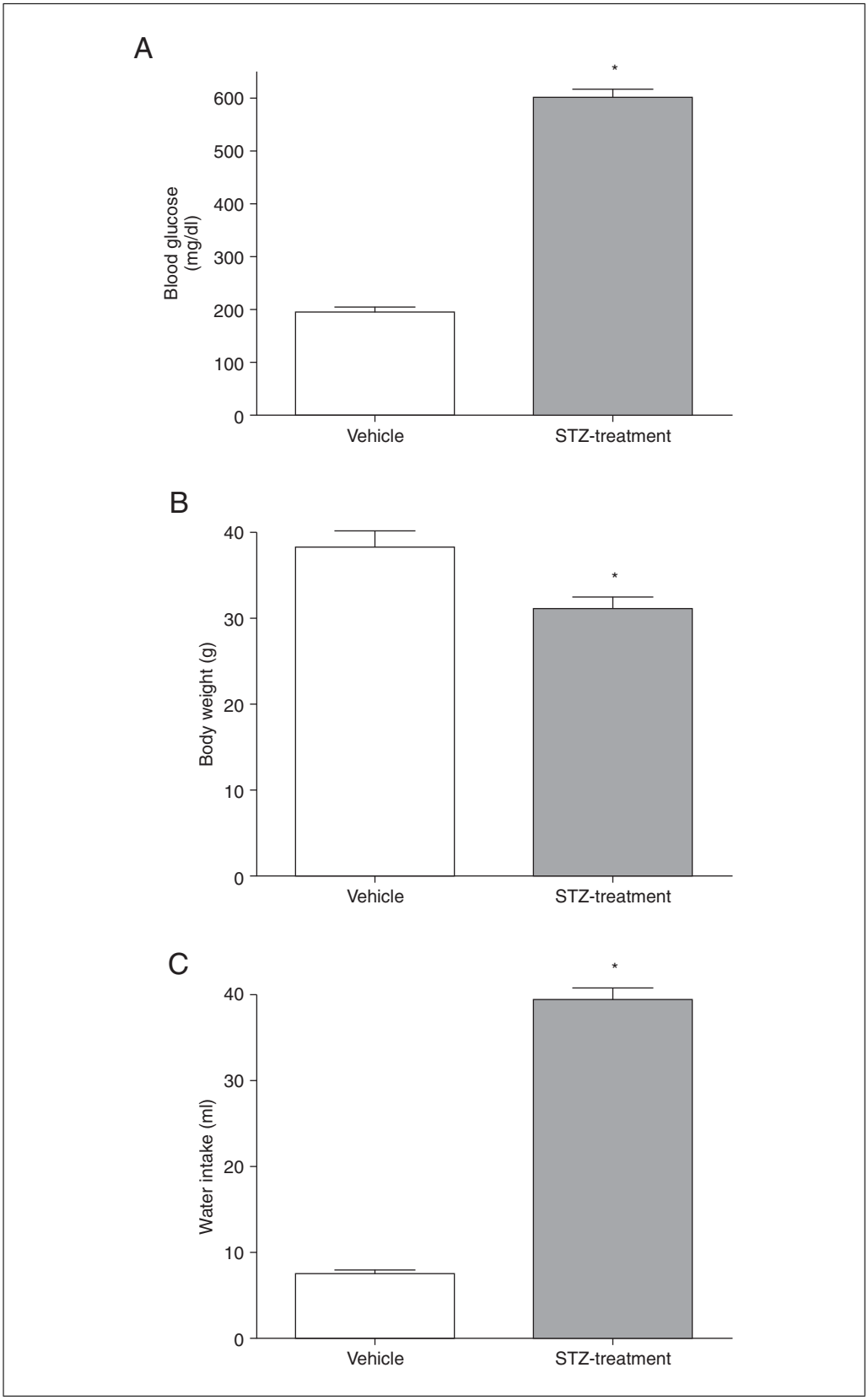

Figure 5.47.2 A single, high dose of STZ causes diabetes in mice ($n = 20$). Mice were treated with 200 mg/kg STZ or sodium citrate buffer vehicle (control). The effect of STZ on (**A**) nonfasting blood glucose level, (**B**) body weight, and (**C**) daily water intake at 3 weeks after the STZ injection. Data represent the mean ± SEM. *$P < 0.001$ versus control.

BASIC PROTOCOL 2

STREPTOZOTOCIN-INDUCED TYPE 1 DIABETES MELLITUS IN RATS

The rat is commonly used as an STZ-induced diabetic model. As in the mouse, the production of a diabetic state in rats is dependent on the dose of STZ (Arison et al., 1967; Junod et al., 1969; Ganda et al., 1976). The most frequently used procedure is to administer one dose of STZ (40 to 70 mg/kg) to rats aged 8 to 10 weeks (Brondum et al., 2005). Many investigators use a single dose of approximately 65 mg/kg to establish diabetes using the procedure described in this protocol.

This protocol describes administration of a single dose of STZ (65 mg/kg, i.p.) to rats to generate a T1DM state. The diabetic rats can be used to study the pathogenesis of T1DM, as well as to evaluate antidiabetic agents (Bond et al., 1983).

Materials

Sprague-Dawley or Wistar male rats: 150 to 200 g, 8 to 10 weeks old (Charles River Breeding Laboratories); 10 to 16 per treatment group recommended
Standard rodent chow diet (Harlan)
50 mM sodium citrate buffer (enzyme grade; Fisher), pH 4.5: prepared just before use
Streptozotocin (STZ; Sigma)
10% (w/v) sucrose (Sigma): prepared just before use
Test compound(s)

Rodent cages
Temperature-, humidity-, and light-controlled housing
1.5-ml microcentrifuge tubes
Aluminum foil
1-ml syringes
23-G needles
One Touch Basic blood glucose monitoring system (Lifescan)

Additional reagents and equipment for injection of rats (Donovan and Brown, 2006a), blood collection from rats (Donovan and Brown, 2006b), and anesthesia of rats (Donovan and Brown, 1998)

Prepare animals

1. At least 5 days prior to the start of the experiment, house two to five male rats per cage at 24°C ± 1°C and 55% ± 5% humidity, with a 12-hr light-dark cycle (light on at 8:00 and light off at 20:00) Allow the rats free access to food and water.

   Because female rats are less sensitive to STZ, most investigators use only males.

   The protocol described here minimizes variability, but it is recommended that group sizes of 10 to 16 be used. These high numbers per group will allow for the anticipated morbidity and variance. Usually >80% of STZ-injected rats develop diabetes in an experiment.

2. Weigh all rats accurately to 1 g and randomly divide them into control and experimental groups.

   The number of rats should be equal for each group.

3. On experimental day 1, fast all rats for 6 to 8 hr prior to STZ treatment. Provide water as normal.

Treat animals with STZ

4. Weigh 32.5 mg STZ into a 1.5 ml microcentrifuge tube and cover the tube with aluminium foil; use one tube for each rat. Prepare the citrate buffer.

5. Immediately prior to injection, dissolve STZ in the 50 mM sodium citrate buffer (pH 4.5) to a final concentration of 32.5 mg/ml.

   The STZ solution should be prepared freshly for each injection and injected within 5 min of being dissolved.

6. Using a 1-ml syringe and 23-G needle, inject the STZ solution i.p. (Donovan and Brown, 2006a) at 65 mg/kg (2.0 ml/kg) for the study group. Inject an equal volume of citrate buffer (pH 4.5) i.p. for the control group. Alternatively, using a 1-ml syringe and 25-G needle, inject the STZ solution i.v. at 65 mg/kg (2.0 ml/kg) for the study group. Inject an equal volume of citrate buffer (pH 4.5) i.v. for the control group.

   Intravenous injection should be undertaken using brief anesthesia with isoflurane or some other suitable inhalation anesthetic (Donovan and Brown, 1998). The dorsal vein of the penis or saphenous vein can be used. Intravenous injection increases the success rate for the induction of diabetes. Anesthesia is not required for intraperitoneal injection.

7. Return the rats to their cages. Provide normal food and 10% sucrose water.

8. On experimental day 2, switch the 10% sucrose water to regular water.

9a. *For studies involving early-stage T1DM:* On experimental day 10, fast all rats for 6 to 8 hr (between 7 a.m. and 1 to 3 p.m.). Test the blood glucose level from a tail vein blood sample (Donovan and Brown, 2006b) using a One Touch Basic blood glucose monitoring system to check hyperglycemia.

   If the diabetic animals are for assessing early-stage mechanisms of T1DM or for screening compounds for treatment of early-stage diabetes, the models are validated for further study when hyperglycemia is established in the STZ-injected rats [i.e., blood glucose levels are >150 mg/dl (8.3 mmol/liter) and/or statistically higher compared to control rats]. In this case, skip step 9b. If the diabetic animals are ***not*** *for assessment of early-stage T1DM mechanisms, or are for screening potential compounds not intended for early treatment of diabetes, skip step 9a.*

   If <40% of the STZ-injected rats attain a diabetic state, re-test blood glucose levels as described in step 9b.

   Usually, determination of blood glucose levels is sufficient to diagnose diabetes, so it is unnecessary to measure insulin levels.

9b. *For re-test of groups failing the first test for diabetes or for studies involving later-stage T1DM:* On experimental day 21, fast all mice for 6 to 8 hr (e.g., from 7:00-13:00 or 15:00). Test the blood glucose level from a tail vein blood sample (Donovan and Brown, 2006b) using a One Touch Basic blood glucose monitoring system to confirm STZ injection-induced hyperglycemia.

   Fasting glucose levels for mild hyperglycemia should be >150 mg/dl (8.3 mmol/liter) and/or exhibit statistically significant increases in the STZ-injected mice compared to control mice.

   Usually at week 3, most STZ-injected rats develop severe diabetes with blood glucose levels typically >250 to 600 mg/dl (13.9-33.3 mmol/liter). If >60% of STZ-injected rats still do not exhibit mild hyperglycemia, check whether there are any problems in the experiment (see Critical Parameters and Troubleshooting).

10. If a test agent or compound is being assessed for its ability to correct hyperglycemia, extend the protocol longer, depending on the intent of the experiment. Treat groups of animals as described in steps 3 to 9 to establish a diabetic state, and then treat the animals with the potential restorative therapy. Include groups that receive appropriate vehicle injections as controls.

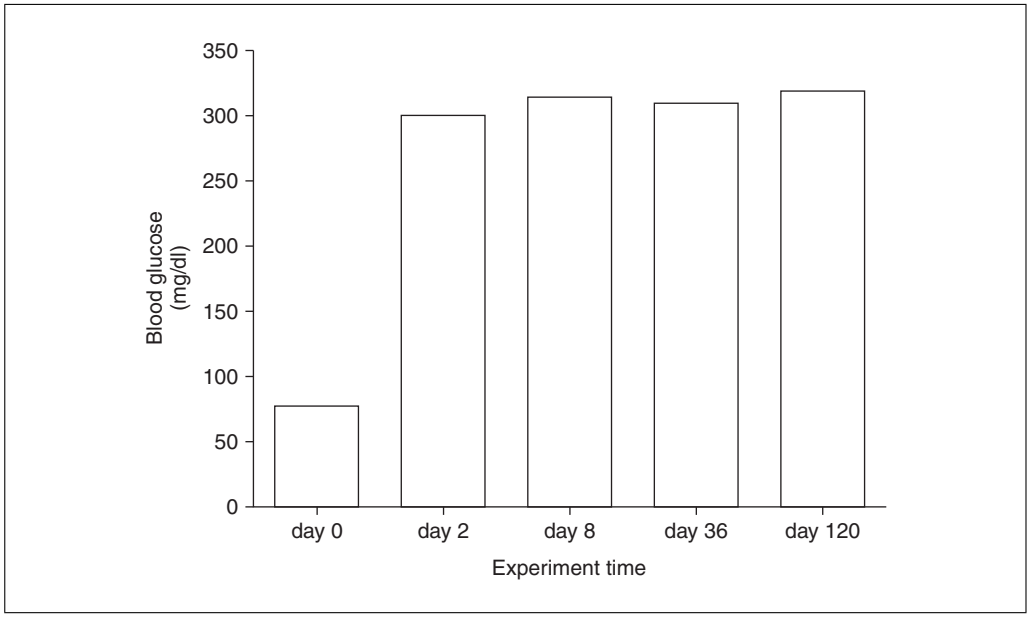

Figure 5.47.3 STZ-induced hyperglycemia in rats. A single 65 mg/kg dose of STZ causes hyperglycemia in rats. Fasting blood glucose levels were monitored before and after the STZ injection on the indicated days.

The length of the experiment depends on the purpose of the investigation, e.g., from several days for acute studies to several weeks for studies on diabetic complications.

Figure 5.47.3 illustrates rat blood glucose changes after STZ (65 mg/kg) injections on different days.

BASIC PROTOCOL 3

THE STREPTOZOTOCIN-NICOTINAMIDE RAT MODEL

This model uses concurrent administration of nicotinamide to afford partial protection of β-cells against STZ (Masiello et al., 1998). It is based on the work of Junod et al. (1969), who systematically investigated the early demonstration (Schein et al., 1967) that nicotinamide protected against the diabetogenic effect of STZ. This regimen produces a model of insulin-deficient, but not insulin-resistant, T2D, characterized by stable, moderate hyperglycemia, associated with 60% loss of β-cell function (Masiello et al., 1998; Ghasemi et al., 2014).

Materials

Sprague-Dawley or Wistar male rats: 150 to 200 g, 8 to 10 weeks old (Charles River Breeding Laboratories); 10 to 16 per treatment group, recommended
Standard rodent chow diet (Harlan)
Nicotinamide (Sigma)
0.9% (w/v) sodium chloride
50 mM sodium citrate buffer (enzyme grade; Fisher), pH 4.5: prepared immediately before use
Streptozotocin (STZ; Sigma)
Test compound(s)

Rodent cages
Temperature-, humidity-, and light-controlled housing
1-ml syringes
23- and 25-G needles
One Touch Basic blood glucose monitoring system (Lifescan)

Additional reagents and equipment for injection of rats (Donovan and Brown, 2006a), blood collection from rats (Donovan and Brown, 2006b), and anesthesia of rats (Donovan and Brown, 1998)

Prepare animals

1. At least 5 days prior to the start of the experiment, house two to five male rats per cage at 24°C ± 1°C and 55% ± 5% humidity, with a 12-hour light-dark cycle (light on at 8:00 and off at 20:00). Allow the rats to have free access to food and water.

   Males are generally preferred for these studies, as female rats are less sensitive to STZ.

   While the protocol is designed to minimize variability, it is recommended that group sizes number 10 to 16 animals each. This allows for the morbidity and variance generally associated with these studies. Usually >80% of STZ-injected rats develop diabetes under this protocol.

2. Weigh all rats accurately to 1 g, and randomly divide them into control and experimental groups.

   The number of rats should be the same in each group.

3. On experimental day 1, fast all rats for 6 to 8 hr (from 7:00 to 13:00-15:00) prior to STZ treatment. Provide water as normal.

Induce diabetes with STZ and nicotinamide

4. Dissolve nicotinamide in 0.9% sodium chloride solution to a concentration of 230 mg/ml.

5. Weigh 32.5 mg STZ into a 1.5 ml microcentrifuge tube and cover the tube with aluminium foil; use one tube for each rat. Prepare the citrate buffer.

6. Using a 1-ml syringe and a 23-G needle, inject nicotinamide i.p. (Donovan and Brown, 2006a) at a dose of 230 mg/kg (1.0 ml/kg).

   The nicotinamide injection must be made 15 min before the i.v. administration of streptozotocin.

7. Immediately prior to injection, dissolve STZ in the 50 mM sodium citrate buffer, (pH 4.5 (see step 5), to a final concentration of 32.5 mg/ml.

   The STZ solution should be prepared fresh for each injection and administered within 5 min of dissolution.

8. Using a 1-ml syringe and 25-G needle, inject the STZ solution i.v. (Donovan and Brown, 2006a) at 65 mg/kg (2.0 ml/kg) for the experimental group. The control animals receive an i.v. injection of an equal volume of citrate buffer (pH 4.5) only.

   Intravenous injections should be performed while the animal is anesthetized with a short-acting agent such as isoflurane or some other inhalation anesthetic (Donovan and Brown, 1998). The dorsal vein of the penis or saphenous vein can be used.

9. Return the rats to their cages. Provide normal food and drinking water.

10. At around 8:00 a.m. on experimental day 10, test the blood glucose level from a tail vein blood sample (Donovan and Brown, 2006b) using a One Touch Basic blood glucose monitoring system.

    While the blood glucose concentrations should be >150 mg/dl (8.3 mmol/liter), large inter-laboratory variation has been reported (Ghasemi et al., 2014). For this reason every laboratory must set its own criterion, although animals with a blood glucose concentration of less than 150 mg/dl would be excluded. Masiello (2006) reports that, using this procedure, 75% to 80% of animals develop moderate non-fasting hyperglycemia,

with the remaining animals either becoming severely hyperglycemic at 2 to 3 weeks or remaining normoglycemic but with impaired glucose tolerance. The same protocol can be used for mice. The dose of STZ and the time between administration of nicotinamide and STZ are critical. Insulin deficiency will be greater if the dose of STZ is too high or the time delay between the administration of nicotinamide and STZ is too long.

Treat animals with test agent(s)

11. If a test agent is being assessed for its ability to correct hyperglycemia, the diabetic animals can usually be maintained for several weeks. For testing of drug candidates, treat animals as described in steps 3 to 11 to establish the diabetic state and with the candidate drug, with vehicle injections in control subjects.

BASIC PROTOCOL 4

THE FAT-FED STREPTOZOTOCIN RAT MODEL

While the nicotinamide-STZ rat or mouse provides a model for insulin-deficient T2D, most patients with T2D display insulin resistance in addition to impaired insulin secretion. A model with these characteristics is created by administering moderate doses of STZ to animals rendered insulin resistant by prior consumption of a high-fat diet. This produces hyperglycemia, associated with hyperinsulinemia and insulin resistance (Reed et al., 2000).

Materials

Sprague-Dawley or Wistar male rats: 150 to 200 g, 8 to 10 weeks old (Charles River Breeding Laboratories), 10 to 16 per treatment group
A high-fat diet: 60% fat by caloric content (D12492 diet; Research Diets, *http://www.researchdiets.com/opensource-diets/diet-induced-disease-models/obesity*)
A low-fat diet from the same supplier for control animals (with the only difference between the diets being the % of the caloric intake provided by fat)
50 mM sodium citrate buffer (enzyme grade; Fisher), pH 4.5: prepared immediately before use
Streptozotocin (STZ; Sigma)
Test compound(s)

Rodent cages
Temperature-, humidity-, and light-controlled housing
1-ml syringes
23-G needles
One Touch Basic blood glucose monitoring system (Lifescan)

Additional reagents and equipment for injection of mice (Donovan and Brown, 2006a) and blood collection from mice (Donovan and Brown, 2006b)

Prepare animals

1. At least 5 days prior to initiating the experiment, house two to four male rats per cage at 24°C ± 1°C and 55% ± 5% humidity, with a 12-hr light-dark cycle (light on at 8:00 a.m. and off at 8:00 p.m.). Allow free access to food and water.

   While the protocol is designed to minimize variability, it is recommended that groups number 10 to 16 animals each. This allows for the morbidity and variance generally associated with these studies. Usually >80% of STZ-injected rats develop diabetes under this protocol.

2. Weigh all rats accurately to 1 g and randomly divide them into control and experimental groups.

   The number of rats must be the same for each group.

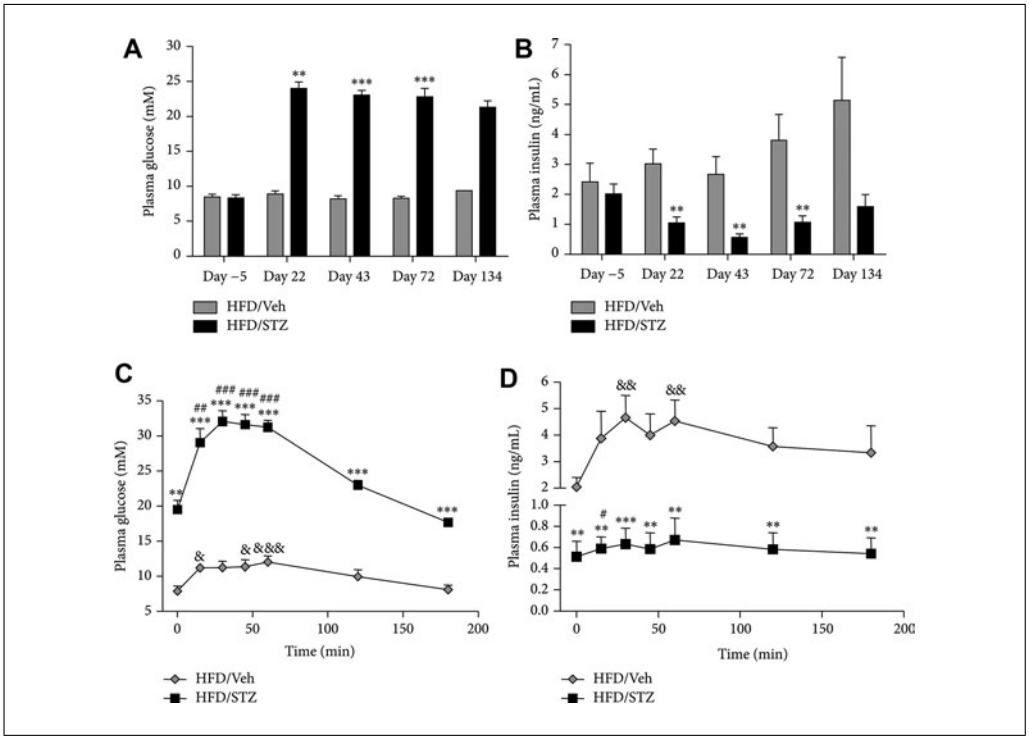

Figure 5.47.4 Effects of HFD/STZ on glucose and insulin levels. Time course of the effects of HFD/STZ ($n = 12$ rats) on (**A**) fasting plasma glucose and (**B**) fasting plasma insulin, in comparison with HFD/Veh treatment ($n = 6$ rats). At day 120, an oral glucose tolerance test (OGTT) was performed and plasma glucose (**C**) and plasma insulin (**D**) were quantified in the two groups of rats. Data are mean ± SEM. Comparisons between HFD/STZ and HFD/Veh rats were performed with a Mann-Whitney test: ∗, $P < 0.05$; ∗∗, $P < 0.01$; and ∗ ∗ ∗, $P < 0.001$. For further analysis of the OGTT, comparisons to baseline values in each group were made using a Friedman test with Dunn's post hoc test: HFD/Veh &, $P < 0.05$; &&, $P < 0.01$; and &&&, $P < 0.001$; HFD/STZ #, $P < 0.05$; ##, $P < 0.01$; and ###, $P < 0.001$. Modified from Byrne et al., (2015) with permission of Professor Victoria Chapman.

3. Place rats on the high-fat diet for 3 weeks. If one aim of the study is to compare the effects of STZ plus the HFD with STZ alone, include a group of controls that receive a low-fat laboratory chow diet from the same vendor.

4. On day 22, fast all rats for 6 to 8 hr (from 7:00 a.m. until 1:00-3:00 p.m.) prior to STZ treatment. Provide water as normal.

Treat animals with STZ

5. Weigh 40 mg STZ into a 1.5 ml microcentrifuge tube and cover the tube with aluminum foil. Prepare the citrate buffer.

6. Immediately prior to injection, dissolve STZ in the 50 mM sodium citrate buffer (pH 4.5) to a final concentration of 40 mg/ml.

   The STZ solution should be prepared immediately before injection and administered within 5 min of dissolution.

7. Using a 1-ml syringe and 23-G needle, inject STZ i.p. (Donovan and Brown, 2006a) into the experimental group at 40 mg/kg (1.0 ml/kg). Inject an equal volume of citrate buffer (pH 4.5) intraperitoneally into the control animals.

   This dose of STZ should produce a stable hyperglycemia in the high-fat diet rat for at least 130 days. The dose of STZ is critical. If it is too large, it yields a model that more closely resembles T1. Zhang et al. (2008) advocate the use of two lower doses of STZ (30 mg/kg, i.p.) administered at weekly intervals. Under this treatment regimen, 85%

of the animals develop diabetes with a mean fasting blood glucose of ~14 mmol/liter (~252 mg/dl).

8. Return the rats to their cages. Provide the high-fat or control diet food as before and normal drinking water.

9. At around 8:00 a.m., 10 days after STZ administration, measure the blood glucose level in a tail-vein blood sample (Donovan and Brown, 2006b) using a One Touch Basic blood glucose monitoring system.

   The STZ animals should have a blood glucose >15 mmol/liter (270 mg/dl).

10. The animals can generally be maintained as hyperglycemic for several weeks for the testing of antihyperglycemic drug candidates. Treat groups of animals as described in steps 3 to 10 to establish the diabetic state, after which initiate the administration of test compounds to measure the hypoglycemic response in comparison to diabetic animals treated with vehicle only.

    Displayed in Figure 5.47.4 are data from rats made diabetic using this protocol, demonstrating the stability of hyperglycemia across 134 days, and the profound impairment of glucose-induced increases in plasma insulin concentrations during an oral glucose tolerance test conducted after 120 days.

COMMENTARY

Background Information

Described in this unit are methods for using STZ to selectively destroy pancreatic islet β-cells in mice and rats to generate animal models of T1DM and T2D. The T1DM animals can develop diabetic complications, e.g., diabetic neuropathy (Usuki et al., 2007), diabetic nephropathy (Breyer et al., 2005), and diabetic atherosclerosis (Wu and Huan, 2007). The models are used not only to study the pathological consequences of T1DM, but also to assess and evaluate experimental approaches for the treatment of this condition, in particular therapeutic approaches for reducing hyperglycemia. Rat and mouse models of diabetes have distinct advantages over other species, including the size of the animals, short induction period, ease of inducing the condition, and cost effectiveness (Wu and Huan, 2007).

Multiple, low-dose STZ-induced diabetic mouse models may more closely resemble human T1DM than models in which hyperglycemia is induced by a single large dose of the toxin, because of the association of hyperglycemia with lymphocytic infiltration of the pancreatic islets, marked β-cell apoptosis, insulitis, and insulin deficiency (Like and Rossini, 1976; Bonnevie-Nielsen et al., 1981; Kolb, 1987; Weide and Lacy, 1991). Moreover, as there is evidence for the contribution of autoimmunity in this model, it is much more suitable for studying the underlying pathogenesis of T1D than the high-dose STZ models where direct, toxin-induced necrosis of the β-cell is the predominant mode of cell death (Lin et al., 2010). However, because STZ may be toxic to organs and tissues other than the pancreatic islet β-cells, STZ models do not precisely mimic the human condition. For this reason, extrapolation of the findings directly to humans is not always possible. This is particularly true when using a single, high dose of STZ, which directly destroys β-cells rapidly and completely, therefore lacking some features of T1DM, such as pancreatic insulitis (Kolb, 1987).

While T2D is the predominant form of this condition in humans, the challenges in developing an animal model for it are greater than for T1DM. Genetic models, especially the Zucker diabetic fatty rat and the db/db mouse, perhaps come closest to resembling the human disease. Nonetheless, their use is limited because they display some important differences from the human condition (Wang et al., 2014), and they are very expensive. Detailed in this unit are two T2D models that are used most often. The nicotinamide STZ rat, a model for non-insulin-dependent, insulin-deficient T2D, is limited in not being insulin-resistant, a major feature of most human cases. The use of high-fat feeding to induce insulin resistance, followed by low-to-moderate doses of STZ to produce mild to moderate insulin deficiency, may currently be the most useful of the T2D models. The high-fat model is generally considered the best for characterizing many of the complications associated with human diabetes.

As none of these models precisely mimic human T1D or T2D, the choice of a model depends on the aim of the study. The models described in this unit are useful for evaluating potential anti-diabetic agents, as well as for studying diabetes-induced long-term complications. They are more limited, however, as tools for defining the etiology of the condition, although the multiple low-dose STZ mouse model may be of some value in this regard.

Critical Parameters and Troubleshooting

General comments

While Basic Protocols 1 (along with its Alternate Protocol) and 2 are generally accepted as established procedures for studying T1DM, a consensus has yet to form on the best protocols for the STZ-nicotinamide and the STZ combined with a high-fat diet models for T2D. For the nicotinamide-STZ model, the main variables are the doses of nicotinamide (60 to 290 mg/kg, i.p.) and streptozotocin (45 to 65 mg/kg, i.p. or i.v.) employed, although there is general agreement on the time interval (15 min) between injection of the two agents (Ghasemi et al., 2014). In the case of the STZ/high-fat model, there is variability in the % fat in the diet (40% to 60%), the duration of the high-fat diet before STZ injection (2 to 12 weeks), the inclusion (or not) of sucrose, and the dose and route of administration of STZ (15, 35, or 50 mg/kg i.v., or 25 to 50 mg/kg, i.p.; Skovso, 2014). Indeed, in some cases, STZ was administered to neonates followed by the high-fat diet (e.g., Mancini et al., 2013).

STZ stability

Streptozotocin should be stored at −20°C to avoid desiccation. After weighing, the microcentrifuge tube containing the sample of STZ must be covered with aluminum foil to protect it from light. As STZ is unstable in solution, even at an acidic pH, it must not be mixed into citrate buffer until immediately prior to injection. The STZ solution should be prepared fresh and injected within 5 min of being dissolved because it decomposes in citrate buffer within 15 to 20 min. Although Ghasemi et al. (2010) suggest that STZ solutions may not be as unstable as previously believed, to reduce variability it is best to administer it within 5 min of its preparation.

Animal gender sensitivity to STZ

There is a strong influence of gender on the development of diabetes in laboratory animals. While females are resistant to the effects of low-dose STZ, this can be overcome by increasing the dose (Kolb, 1987). Because male pancreatic islet β-cells are more prone than female to STZ-induced cytotoxicity, male subjects are more popular for study (Kolb, 1987). Although the precise reason for this gender difference remains undefined, estrogens are known to reduce the sensitivity of male rats to STZ-induced diabetes (Paik et al., 1982). Notably, however, the diabetes produced by a high dose of STZ (95 mg/kg) in rats protected by nicotinamide is more severe in female than in male rats (Vital et al., 2006).

Animal strain sensitivity to STZ

Different strains of animals display different sensitivities to STZ. For mice, CD-1 and C57BL/6 are reliably sensitive to this toxin (Like and Rossini, 1976; Rossini et al., 1977), as are Sprague-Dawley and Wistar rats. In contrast, Balb/cJ are resistant to the diabetes-inducing effects of multiple low doses of STZ (Zunino et al., 1994). If blood glucose levels fail to rise above 150 mg/dl by weeks 3 or 4 after terminating STZ administration, it is possible the animal strain being employed is insensitive to this toxin.

Fatal hypoglycemia after STZ injection

Some animals die quickly after STZ treatment due to massive islet β-cells necrosis and a sudden release of insulin that results in fatal hypoglycemia usually within 48 hr of STZ injection. To prevent this, it is best to routinely provide animals with 10% sucrose water after STZ treatment (see *http://www.AMDCC.org*). If the number of animal deaths is >20% when using the single, high-dose STZ diabetic mouse protocol, treat the animals with 1 ml of 5% glucose solution i.p. 6 hr after STZ injection instead of providing 10% sucrose water (Huang and Wu, 2005). To avoid fatal hypoglycemia in the multiple, low-dose STZ-treated diabetic mice, provide 10% sucrose water for 6 days, beginning on experimental day 1. If mortality is high (>20%) in the single-dose, STZ-treated diabetic rats, provide 10% sucrose water for 2 days after the STZ injection. Generally, severe hypoglycemia is more likely to occur after high, single doses of STZ.

Fasting and nonfasting blood glucose levels

Because mice and rats are nocturnal feeders, an overnight fast before measuring blood glucose levels usually translates into a fast of ~24 hr. This 24-hr fast can activate several

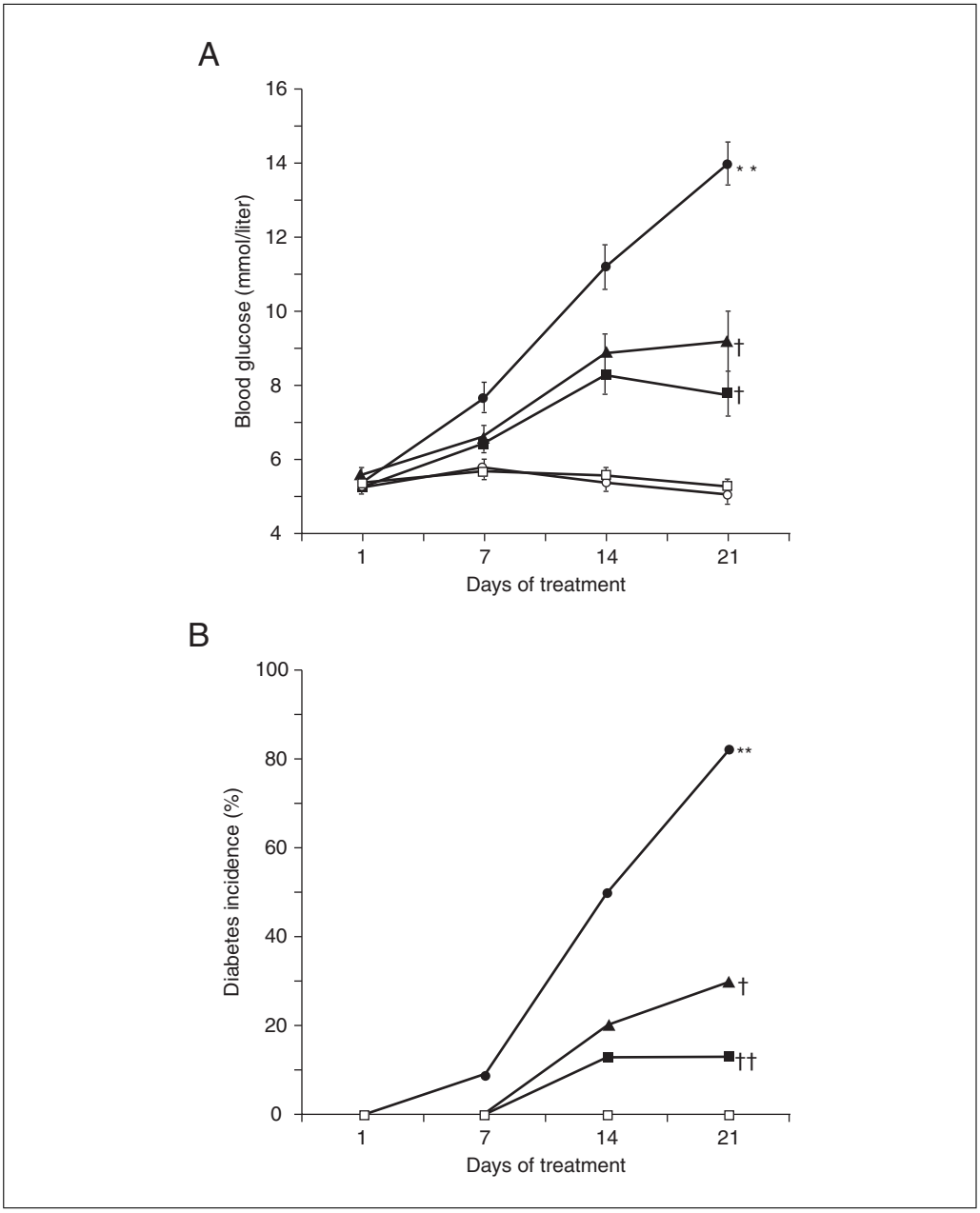

Figure 5.47.5 Effect of daily treatment with inosine on STZ-induced diabetes in mice. Daily treatment with 100 or 200 mg/kg inosine for 21 days decreased hyperglycemia (**A**) and incidence of diabetes (**B**) following multiple, low-dose STZ (MLDS) treatment of the mice. Mice were either untreated (open circles); given daily doses of 200 mg/kg inosine alone (open squares); or treated with STZ (on days 1 to 5) in combination with vehicle (filled circles), 100 mg/kg inosine (filled triangles), or 200 mg/kg inosine (filled squares) starting on day 1. Diabetes incidence is expressed as a cumulative percentage of mice with a blood glucose ≥11 mmol/liter. Results are means ± SE for $n = 20$ mice in two separate experiments with 10 mice per experimental group. **, $P < 0.01$ compared with vehicle-treated mice; †, $P < 0.05$; ††, $P < 0.01$ compared with MLDS-treated mice. Reproduced from Mabley et al. (2003) with permission from The Feinstein Institute for Medical Research.

physiologic responses that obscure the reliability of glucose readings. Because of this, fasting should be initiated on the morning of blood sampling. The National Institutes of Health (NIH) and the Animal Models of Diabetic Complications Consortium (AMDCC) have established a protocol of fasting mice from 7 a.m. to 1 p.m., with blood drawn at 1 p.m. (Breyer et al., 2005; *http://www.AMDCC.org*). Similarly, the accepted rat fasting time is 6 to 8 hr, between 7 a.m. and 1 to 3 p.m., after which the blood sample is taken for glucose analysis.

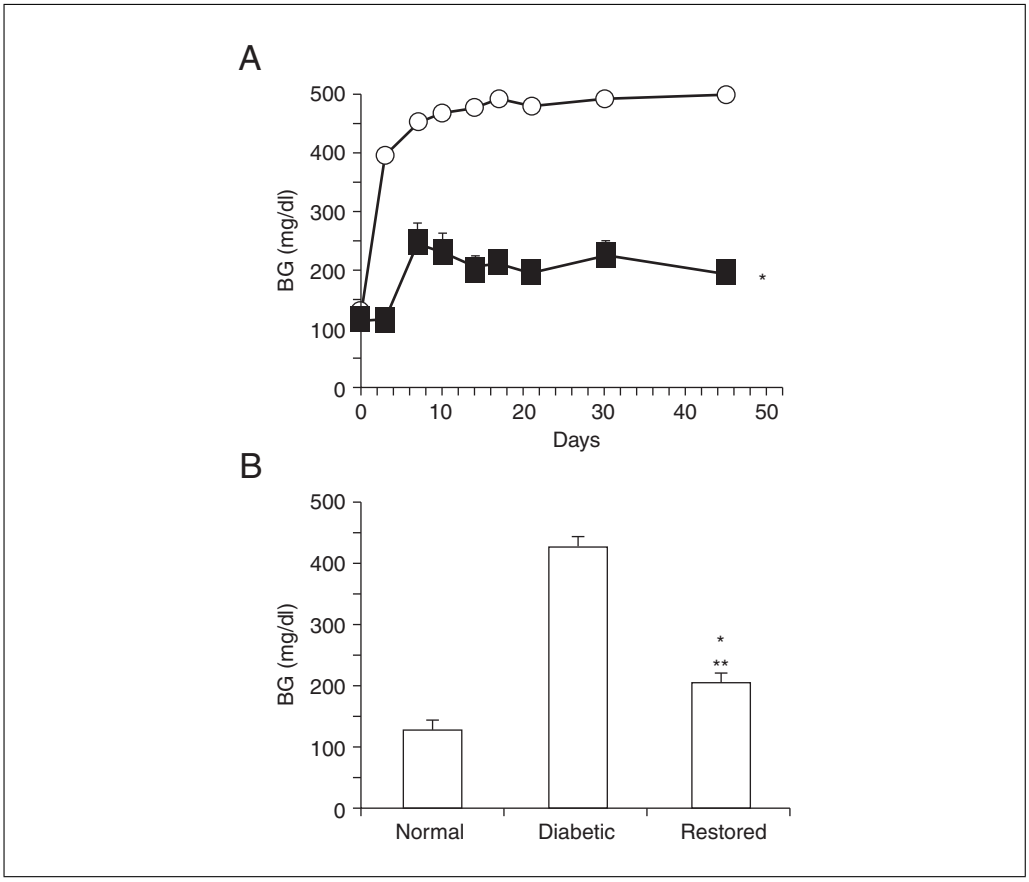

Figure 5.47.6 Restoration of β-cell function in STZ-induced diabetic mice by transplantation of syngeneic islets under one kidney capsule. (**A**) Blood glucose (BG) levels of single high-dose STZ-induced diabetic (open circles) or restored (filled squares) mice. (**B**) Mean BG levels (±SEM) were calculated from each group. * and ** indicate statistical differences (ANOVA) between the restored ($n = 14$) and the normal ($n = 10$) or diabetic ($n = 34$) groups, respectively. Reproduced from Yin et al. (2006) with permission from the American Diabetes Association.

Blood glucose levels between fasting and nonfasting animals are quite different. The absolute levels of blood glucose in a fasting state are lower and less variable than in a nonfasting state. There is no standardized hyperglycemia level for mice or rats because different institutes and investigators use different, nonstandardized, fasting and nonfasting methods. However, there are three key points of general agreement: (1) use the same approach to test blood glucose levels for both control and STZ-treatment groups in the same experiment, e.g., either a non-fasting state or a fasting state for both control and STZ-treatment groups; (2) never use fasting and non-fasting states in the same experiment; and (3), hyperglycemia is defined as a blood glucose level in the STZ-treatment groups that is significantly higher than in the control groups.

Generally speaking, the blood glucose level in the STZ-treatment groups for nonfasting hyperglycemia should be >200 mg/dl (11.1 mmol/liter), whereas for fasting diabetic animals the blood glucose should be >150 mg/dl (glucose of 18 mg/dl = 1 mM). The most important point is that there should be a statistically significant difference between the STZ-treatment and control groups. Usually, by 3 weeks after STZ injection, more than 50% of the animals develop severe hyperglycemia, with blood glucose levels attaining levels of 300 to 600 mg/dl (Deeds et al., 2011). If the study involves a chronic condition or diabetic complications (e.g., STZ-induced diabetic atherosclerosis) using multiple, low-dose STZ-induced diabetic mice, a second round of STZ injections is needed at week 7 to ensure maintenance of the diabetic state.

Anticipated Results

For diabetic mice, typical time-dependent daily changes of glucose and insulin secretion are summarized in Figure 5.47.1. The figure illustrates glucose and insulin secretory responses to multiple, low doses of STZ in treated mice. Usually, the blood glucose

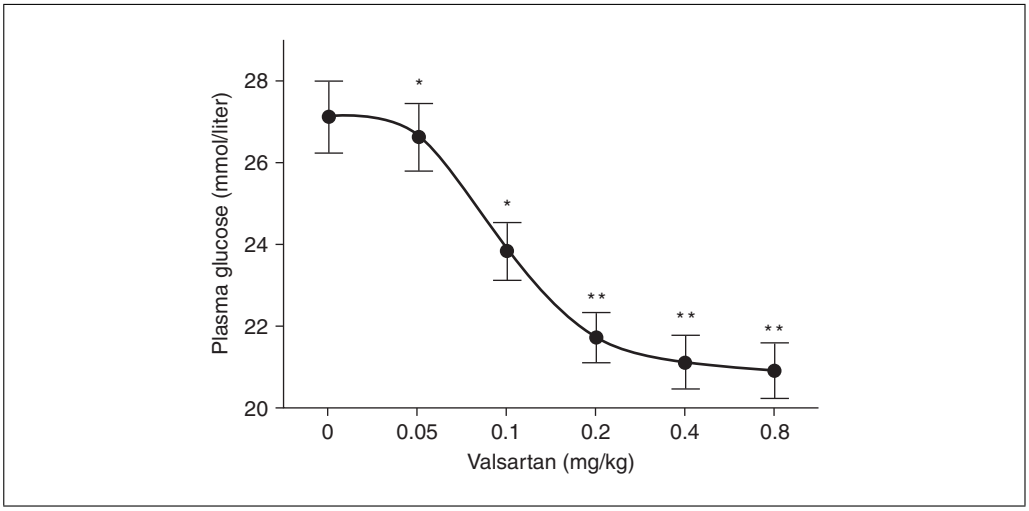

Figure 5.47.7 Effect of valsartan on plasma glucose concentration in STZ-induced diabetic rats. The diabetic rats were treated with valsartan 2 weeks after STZ injection. Values of mean ± SE were obtained from each group of eight animals. *, $P < 0.05$; **, $P < 0.01$ versus data from animals treated with vehicle (0 mg/kg valsartan). Reproduced from Chan et al. (2003) with permission from Lippincott, Williams, & Wilkins.

levels in the STZ-injection groups are significantly higher than in the control groups on experimental day 10. By experimental weeks 3 to 4, blood glucose levels indicate severe hyperglycemia (300 to 600 mg/dl; 16.7 to 33.3 mmol/liter) in ~50% of STZ-treated animals. Insulin levels are low, although this parameter is not normally measured. Besides glucose and insulin level changes, mice also display, within 3 weeks of STZ treatment, typical T1DM features such as a loss of body weight and polydipsia (Flood et al., 1990). Illustrated in Figure 5.47.2 are the typical features of mouse diabetes. In diabetic rats, hyperglycemia lasts for months (Arison et al., 1967). Shown in Figure 5.47.3 are the typical features of diabetes in rats.

The diabetes models described in this unit can be used to assess diabetic mechanisms, screen compounds, or evaluate therapeutic options. For example, inosine, an immunomodulator/anti-inflammatory agent, significantly reduces blood glucose in the multiple low-dose STZ-induced diabetic mouse model (Fig. 5.47.5; Mabley et al., 2003). In the single high-dose STZ-induced mouse model, syngeneic islet transplantation partially restores β-cell function and corrects hyperglycemia (Fig. 5.47.6; Yin et al., 2006). The STZ-induced diabetic rat model has also been used to evaluate antidiabetic drugs with, for example, valsartan (an angiotensin II receptor antagonist), displaying a dose-dependent anti-diabetic effect (Fig. 5.47.7; Chan et al., 2003).

In studies on the high-fat STZ model (Fig. 5.47.4), it has been shown that both linagliptin (3 mg/kg daily) and metformin (200 mg/kg daily) reduce diabetes-induced changes in mechanical pain threshold (Byrne et al., 2015). Rats rendered diabetic using this protocol also develop nephropathy, making this a useful model for studying potential treatments for this condition (e.g., Luo et al., 2009).

Time Considerations

Approximately 1 working day is needed to perform the STZ injections. On the first day of the experiment, mice must fast for 4 hr and rats for 6 to 8 hr before the STZ injection. The citrate buffer can be prepared during the fasting period. The STZ must be prepared immediately before injection to avoid STZ decomposition. To prevent fatal hypoglycemia in single high-dose STZ-induced diabetic mice, treat the animals with 1 ml of 5% glucose i.p. at 6 hr after the STZ injection.

It takes several hours to test the blood glucose levels on the different post-STZ-injection days, depending on the protocol. The amount of time required over subsequent days or weeks depends on the aim of the study.

If the animals are to be used to study diabetic complications (e.g., STZ-induced diabetic atherosclerosis), the STZ treatment must be repeated at experimental week 7. This requires ~1 working day. The animals can then be used for several more weeks or month, depending on the study design.

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Internet Resources

http://www.AMDCC.org

Web site for Animal Models of Diabetic Complications Consortium (AMDCC), providing new animal models of diabetic complications, with the goal of identifying the most appropriate animal models to study the etiology, prevention, and treatment of diabetic complications.