

How to create an experimental diabetes mellitus model?

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Abstract

Diabetes is a metabolic disorder characterized by chronic hyperglycemia. Early treatment is very important in terms of preventing diabetes-related late complications with high treatment costs and increasing the patient's quality of life. In addition to investigating the pathophysiology of the disease studied, animal experiments pave the way for new approaches in treatments. Although there are many methods that can be used when creating a diabetes model, induction of diabetes with alloxan and streptozotocin are the most preferred ones. The aim of this article is to review the available information on diabetes-related methods, common problems and solutions, with known mechanisms of action, dose and time-determined methods.

Keywords

experimental diabetes, method, streptozotocin, alloxan

Introduction

Diabetes Mellitus (DM) ranks 9th among the causes of death in the world, and according to the World Health Organization (WHO), there are approximately 422 million DM patients in the world, and this number continues to increase exponentially. In the study conducted by Turkey Diabetes Epidemiology (TURDEPI) in 2010, the frequency of DM in Turkey was determined as 7.2% between the ages of 20 and 80. According to the Seventh Diabetes Atlas published by the International Diabetes Federation (IDF), it is reported that there are 415 million DM patients as of the end of 2015, and this number will increase by 52% and reach 642 million by 2040 (Anon n.d.).

DM is a functional disorder, including various smooth muscle structures, which is responsible for the regulation of blood sugar, which is frequently seen throughout the world, and which is associated with biochemical and clinical findings such as chronic hyperglycemia, dialipidemia,

due to disorders in carbohydrate, protein and fat metabolism as a result of insufficient production and/or loss of function of the insulin hormone secreted from the pancreas. It is a chronic metabolic disease with chronic complications such as today, it is still unclear whether DM and Covid-19 increase each other's risk factors in viral infections such as Covid-19. However, treatment options for DM patients are limited due to clinical findings in the fight against the disease, and it is generally thought that the risk of infection, which has been extended to Covid-19, is increased (Hartmann-Boyce et al. 2020).

Another problem caused by DM is that it causes an imbalance between clotting factors and fibrinolysis. As a result of this imbalance, a prothrombotic state occurs with an increased thromboembolic risk. In a study conducted in China in the past (Priestnall et al. 2020), it was observed that prothrombin time and D-dimer concentrations were longer in patients with diabetes who were hospitalized due to Covid-19, and in patients who died. Risk tables such as

advanced age, other metabolic diseases, thrombotic complications, and obesity have been observed in DM patients with Covid-19 infection (Apicella et al. 2020).

In this article, general information about diabetes mellitus is presented; Information was given about the methods of experimental diabetes on experimental animals. In addition, by examining the scientific studies on the subject, it is aimed to be a study that includes collective and versatile information for researchers who will work on this subject.

Diabetes mellitus

DM is characterized by chronic/acute hyperglycemia, which develops as a result of absolute or relative insufficiency of insulin secretion and/or dysfunction of secreted insulin; it is a disease that causes deterioration of carbohydrate, protein and lipid metabolisms and accompanying acute/chronic complications (Çiçek et al. 2018).

Insulin is directly and indirectly responsible for regulating glucose metabolism. It does this by binding to receptors in liver, kidney, muscle and adipose tissue and activating the signaling pathway that includes regulatory proteins for which protein kinases are important. Glucose in plasma occurs as a result of dietary sources, the breakdown of glycogen in the liver (glucogenolysis) or the formation of glucose (gluconeogenesis) from other carbon compounds (precursors) such as lactate, pyruvate, amino acids and glycerol in the liver and kidney (Grossmann et al. 2009).

In DM, there is an increase in the production of reactive oxygen species (ROS) with enzymatic glycosylation of proteins and increased degradation of glucose. This increased ROS level may cause oxidative damage to the cell membrane (Demir and Yilmaz 2014).

In addition to acute complications such as Diabetes, Hyperglycemic Hypersomes Condition (HHD) and Diabetic Ketoacidosis (DKA), it can cause serious health problems and death with chronic microvascular and macrovascular complications. Symptoms such as weight loss, polydipsia, polyuria, and polyphagia are observed in diabetic patients. People with diabetes have a higher risk of developing eye, kidney, nerve, cerebrovascular diseases and heart diseases (Anon n.d.).

Chronic hyperglycemia plays an important role in the occurrence of vascular dysfunctions associated with DM, and many mechanisms related to hyperglycemia of these dysfunctions have been clarified (Senthil Kumar et al. 2006; Kitabchi et al. 2009). This mechanism is explained by damage to the vascular tissue, including the polyol pathway, which causes sorbitol and fructose accumulation, protein formation by advanced glycation end products (AGEs), protein kinase C (PKC) and activation of the hexosamine pathway, one of the causes of cardiovascular pathology caused by hyperglycemia. Which of these causes is dominant is still under investigation.

DM is divided into four main groups; type 1 DM (based on genetics), type 2 DM (which progresses with

living conditions and habits), DM due to various specific causes, and gestational diabetes mellitus (GDM) as gestational diabetes. The most common types are type 1 and type 2 (WHO 2021).

Type 2 diabetes is defined as the inability to use existing insulin due to β -cell dysfunction. Considering these conditions, β -cell insufficiency is imitated with insulin resistance and glucose intolerance in type 2 models that are desired to be created. The most frequently generated models include monogenic obese mice with defects in leptin signaling and obesity.

The aim here is to mimic human obesity and create a response deficiency due to leptin signal defect, where Lepob/ob and Lepdb/db are hyperphagic and obese (Daniels Gatward et al. 2021).

Conditions such as impaired immune system functions, inactivity, and hyperlipidemia can be observed in obese models. At the end of an average of 4 weeks, hyperglycemia peaks. Ketoacidosis and mortality are seen with β -cell failure (Lindström 2007). Mice with the Lepob/ob mutation have C57BL6/J, while those with the Lepdb/db mutation have C57BLKS/J. Although in theory these models are structurally well characterized, they cannot be modeled on humans because they are polygenic (Latham et al. 2009).

Experimental diabetes models in animals

Creating Experimental Diabetes Animal models; investigating the course of new drugs to be administered in the human body is very important in terms of possible complications and side effects. In this way, undesirable results can be prevented or necessary studies can be carried out to achieve the desired results.

Experimental DM creation

Medical and surgical methods are frequently used to create an experimental diabetes model in animals. Chemical methods are the most preferred methods in terms of ease of application and cost. Both of them has advantages and disadvantages. Animals such as mice, rats, guinea pigs, hamsters, rabbits, monkeys, pigs, dogs and cats can be used to induce experimental diabetes (Alarcon-Aguilar et al. 2000; van de Maele et al. 2005; Pari and Umamaheswari 2000).

With chemical applications (medical)

Chemicals most commonly used to create experimental diabetes; alloxan and streptozocin (STZ). Diabetes can be induced by parenteral or intraperitoneal (i.p.), subcutaneous (s.c.), intravenous (i.v.) administration of these chemicals. These chemical agents damage the beta cells in the islets of Langerhans in the pancreas and cause a low

insulin or high glycemic picture. Alloxan and its reduction product, dialuric acid, transform into free radicals such as superoxide radical and hydrogen peroxide, cause calcium increase in the pancreatic beta islet and cause toxicity in the islets (Köse and Cayir 2016). Alloxan is less expensive and can induce diabetes in animals with a single dose. Besides, STZ is more stable. When both are compared, STZ is more preferred due to its reduced toxicity. After the diabetes model is created, stable diabetes is followed in animals before starting the experiments. While this can be achieved an average of 72 hours after alloxan injection, 5 to 7 days should be waited after STZ injection. Although there are studies in which diabetes was induced with a single high dose of STZ, it should be noted that a single dose of STZ is insufficient and weak to examine the type 1 diabetes model. Instead, multiple doses will create a more effective and precise model. In Alloxan, a single i.p. dose is sufficient. Although high-dose STZ induces faster than low-dose, it is not carefully selected for mortality and health problems (Daniels Gatward et al. 2021). In STZ administration, female mice are more resistant to the development of diabetes (Saadane et al. 2020). In addition, considering the hormonal changes, even the time of STZ application may be effective in the formation of diabetes (Anderson and Bluestone 2005).

Creating a diabetes model with STZ

STZ, it is an alkylating chemotherapeutic that is obtained by various methods from synthetic nitrosourea-bearing glucopyrazone products extracted from *Streptomyces Achromogenes* species by fermentation and contains a nitrosourea group in its structure (Çiçek et al. 2018).

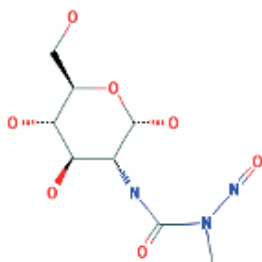


Figure 1. Chemical structure of Streptozocin.

STZ stable conditions are pH 7.4 and 37 °C for an average of 1 hour. Its biological half-life is 5–15 minutes. STZ dissolves well in solvents such as water, alcohol and ketones. It dissolves slowly in polar organic solvents (Çiçek et al. 2018). Nitrosoureas are normally lipophilic, but STZ is hydrophilic despite its nitrosourea structure. This makes it difficult for STZ to be taken into the cell (Lawrence 2009; Eleazu et al. 2013).

Selective toxicity occurs in the beta cells of the pancreas when STZ enters the cell from GLUT2 in the membrane of beta cells through the glucose part in its structure. It causes cell death by forming alkylation in deoxyribonucleic acid (Mythili et al. 2004).

STZ causes cell death in four ways:

1. Methylation of DNA: DNA methylation occurs when STZ is taken into the cell by the GLUT2 carrier in the membrane of beta cells, and as a result, DNA fragments and cell death occurs.
2. Nitric Oxide (NO) Production: STZ acts as a NO donor in pancreatic cells. NO increases guanylate cyclase activity and damages beta cells. In addition, it inhibits the aconitase enzyme and causes DNA to be alkylated and damaged (Eleazu et al. 2013).
3. Production of Reactive Oxygen Species (ROS) by Oxidative Stress: After STZ application, the amount of malonaldehyde, which indicates the oxidative damage of lipids in the membranes, increases, while the amounts of antioxidant enzymes such as catalase, superoxide dismutase (SOD), glutathione peroxidase, glutathione reductase, and catalase decrease. As a result, oxidative stress increases and the cell goes to apoptosis (Çiçek et al. 2018).
4. NF-KB (Nuclear Factor Kappa B) Protein Mediated Cell Signal Change: Another cytotoxicity mechanism of STZ; The NF-KB protein is found in all cell types, under normal conditions, in an inactive state in the cytoplasm and is a transcription factor involved in cell signaling pathways in case of inflammation. With the application of STZ, there is a cell signal change in the NF-KB pathway and the cell goes into apoptosis (Lawrence 2009). STZ selectively inhibits glycoside hydroxylase activity in pancreatic beta cells and causes irreversible O-glycosylation of intracellular proteins in these cells (Konrad et al. 2001; Lee et al. 2010).

High doses of STZ cause necrosis in beta cells, while low doses cause dysfunction of beta cells, thus disrupting the insulin mechanism.

The antineoplastic mechanism of action of STZ in human hepatocellular carcinoma (HepG2) has not been clarified yet, but its effect on (HepG2) cells has been investigated with the MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazol bromide) method, which is used to determine cell viability. In this study, in vitro cancer cells were incubated with 20 mM Stz for 48 hours and a significant decrease in cell number was observed after 48 hours. In the same study, when the STZ concentration was 10 mM, the number of cancer cells decreased by 40%. ROS, nitric oxide (NO) production and lipid peroxidations were increased in HepG2 cells at 10 and 20 mM STZ doses. As a result of the increase in ROS, there was an increase in caspase 3 activity in cells and a decrease in gene expression of Bcl-2, an anti-apoptotic protein, thus apoptosis was observed (Eleazu et al. 2013).

Diabetic dose of STZ differs according to animal species. Doses that produce the maximum diabetic state in various species are indicated: 60–80 mg/kg i.p. in rats. Doses below 40 mg/kg are insufficient to induce diabetes in rats (Katsumata et al. 1992). If the blood glucose level

measured from the tail vein is higher than 200–300 mg/dL two days after STZ administration, these rats are considered diabetic (Kurcer et al. 2007; Etuk 2011; Howarth et al. 2011). If low-dose STZ is administered to adult bioreproductive rats in multiple doses (40 mg/kg, 5 days), a model of autoimmune type 1 diabetes with inflammation can be established (Alcolado and Rees 2005). The diabetes model given a single dose of 60–100 mg/kg STZ does not produce an autoimmune profile (Yu et al. 2000).

This model is a non-insulin dependent diabetes mellitus model. The i.v./i.p dose in mice is 175–200 mg/kg, and the i.v dose in dogs is 15 mg/kg (Eleazu et al. 2013). STZ is prepared freshly by dissolving in 0.1 Molar citrate buffer, pH 4.5. (200 mg/kg) is administered intraperitoneally and it is accepted that diabetes develops in mice within the same day (Öntürk and Özbek 2007; Köse and Cayir 2016) reported that STZ administered to mice intraperitoneally at a dose of 150 mg/kg (single dose) caused experimental diabetes in their diabetes model on rats. After these applications, the serum glucose levels sought to understand whether they have DM are generally 180–500 mg/dL (Etuk 2011).

Stored insulin, which occurs as a result of beta cells damage induced by streptozocin or alloxan, causes hypoglycemia and mortality in the acute period (Gülinnaz and İrer 2004). This event is followed by permanent beta cell damage and loss. An increase in blood glucose level is observed 2 hours after STZ administration to fasted rats and hypoglycemia is observed 6 hours later due to an increase in blood insulin levels. This hypoglycemic state also causes mortality. In order not to interrupt the study and to prevent animal deaths, hypoglycemia should be prevented. Therefore, 5% glucose solution should be given in the first 12–24 hours despite the occurrence of hypoglycemia. Starving animals are more sensitive to the effects of alloxan. The fullness of the animal partially reduces the effect of alloxan. Application of Alloxan or STZ is generally done after 8–12 hours of fasting in experimental protocols (Köse and Cayir 2016).

Creating a diabetes model with Alloxan

Alloxan

Alloxan monohydrate is a uric acid derivative in the structure of [2,4,5,6(1H,3H)- pyrimidinetetrone], hydrophilic, easily soluble in water, should be stored at 2–8 °C in powder form and below 4 °C in solution form. It has been reported that it causes insulin-dependent diabetes by selectively damaging pancreatic beta cells (Öntürk and Özbek 2007).

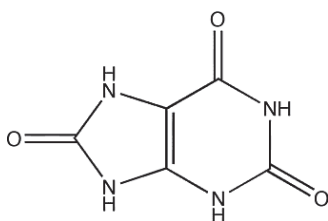


Figure 2. Chemical structure of Alloxan.

The dose required to cause diabetes with alloxan administration is very low and it can cause mortality even if it is slightly higher than the optimum dose to be administered. The toxicity of alloxan in the renal tubule epithelium and the resulting renal failure cause mortality (Lenzen 2008).

DM modeling in rats

Alloxan in rats i.v. dose is 65 mg/kg. Intraperitoneal (i.p.) or s.c. the effective dose should be higher (Federiuk et al. 2004). The most commonly used method is to dissolve 120 mg/kg of alloxan in distilled water or physiological saline (SF) and administer to rats intraperitoneally for three consecutive days. Three days after the last injection of alloxan, the rats are fasted overnight and their blood glucose is measured on the soap. Rats with blood glucose levels above 250 mg/dL are considered DM and participate in the study (Jaouhari et al. 2000). In another method, 150 mg/dL alloxan is dissolved in serum physiology to create experimental DM and i.p. Once administered via the DM route, fasting blood glucose levels are measured, and those above 250 mg/dL are considered DM (Prince et al. 1998). With the application of alloxan, a high amount of insulin is secreted from the pancreas (Öntürk and Özbek 2007). This high insulin level can cause hypoglycemia and can be fatal. To prevent this death, 15–20 mL of 20% glucose solution is administered intraperitoneally 4–6 hours after alloxan administration. Then, in order to prevent hypoglycemia, 5% glucose solution is added to the drinking water of the rats and given for 24 hours. Two weeks later, fasting blood glucose is measured and those with a fasting blood glucose level of 200 to 260 mg/dL participate in the study (Al-Shamaony et al. 1994).

DM modeling in mice

Intraperitoneally administered 150 mg/kg of alloxan dissolved in distilled water or SF (0.9% NaCl) to rats fasted for 18 hours. This process is repeated 3 times, totaling 48. A total of 450 mg of alloxan per kg is administered to a mouse. While injecting, a faint/pronounced pink color may occur in the area where the injector enters, indicating that some of the alloxan solution has leaked out from the injection site. In order to prevent this situation, the syringe should be inserted into the peritoneum in a zig-zag manner. 7 to 10 days after administration, the mice are fasted again for 18 hours and blood glucose levels are measured by drawing blood from their tail veins. Those with fasting blood glucose levels above 180 or 200 mg/dL are considered diabetic mice and included in the study (Alarcon-Aguilar et al. 2000; Yu et al. 2000). After this application, it is expected and possible that some mice will develop DM, some mice will die, and some will not develop DM. Considering this situation, the number of mice in the groups should be kept 30% higher when designing the study.

In another study, it was observed that DM was also observed when 90 mg of alloxan per kg was given to mice in a bolus manner (total dose with a single injection, not gradually) (Kimura et al. 1999).

With surgical applications

Total resection of the pancreas is the most common method used to create a DM model in surgical applications (Acharjee et al. 2013). The result is high, but it is a difficult technique in terms of requiring surgical intervention and creating malabsorption. (Öntürk and Özbek 2007).

By changing genetics

When the DM model is separated into Type1 and Type2 DM by genetic modification, the following figure is formed (Al-Awar et al. 2016).

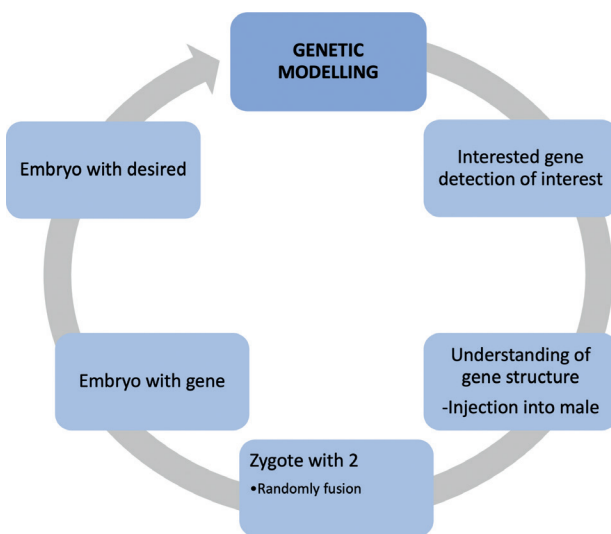


Figure 3. Stages of generating transgenic animal models.

In ob/ob mice, there is an abnormal level of leptin production as a result of a mutation of leptin encoded in chromosome 6, which results in hyperphagia, that is, reduced energy consumption and obesity, resulting in type 2 DM (Pickup and Keen 2002). In db/db mice, the mutation in chromosome 4 causes a defect in the hypothalamic leptin receptor gene, preventing the receptor from responding to leptin.

The Akita mouse, which was first produced in AKITA, Japan, was produced from C57BL/6NSC mouse with inhibition of proinsulin and mutation in the insulin 2 gene. It can be used to investigate macrovascular complications and pathogenesis in neurons in type 1 diabetes (Gurley et al. 2006).

Originally in Japan, Nanobase Diabetic Mouse, NOD, is the preferred model for the pathophysiology of diabetes. Biobreeding Diabetes-Prone and NOD mice, which are spontaneous autoimmune DM models, have islet autoimmunity. These mice show hyperglycemia at 12 and 13 weeks of life and are used for the type 1 model. BB type rats were first described in 1974 at the Biobreeding Laboratories in Ottawa, Canada, and are named after this laboratory, they are divided into subspecies as BBDP/W and BBdp by colonizing with their characteristics (Like et al. 1992). BB rats represent a spontaneous autoimmune diabetes

model and are considered superior to Wistar rats. There is no gender difference between male and female diabetics and they are characterized by hyperglycemia, weight loss, ketonuria. They are obliged to insulin therapy for the continuation of their life cycle (Pandey and Dvorakova 2019).

Goto-Kakizaki rats are hyperglycemic rats, genetic spontaneously diabetic rats selected and bred from healthy non-diabetic Wistar rats. They are used as a Type 2 DM model (Chen and Wang 2005). Another group of type 2 DM rats; Spontaneous single gene mutation and autosomal recessive inheritance; ob/ob and db/db are mice and fa/fa are rats.

LEW rats, which are congenic Lewis rats, represent spontaneous autoimmune type 1 diabetes. There is no difference between males and females and they show the characteristics of diabetes at about 8–9 weeks of age. The biggest advantage of using Lew rats is that they indicate the pre-diabetic period and can be intervened without showing hyperglycemia features. These features are due to the study conducted to prevent the disease before it occurs, rather than the treatment of type 1 diabetes (Pandey and Dvorakova 2019).

Conclusions

As a result of the rapidly increasing prevalence of diabetes mellitus, diabetic rat models are of great importance in terms of shedding light on the macro and micro complications and pathogenesis in patients with diabetes. In addition, models are also very important in terms of new drug development. Although animal models made so far have many advantages, they still have limitations for researchers. For this reason, it is necessary to design and implement much better diabetes mellitus (DM) models for the underlying mechanisms of the problems encountered in modeling, therapeutic approaches to diabetes and possible complications.

When designing a diabetes model, the following should be considered: having micro and macro complications, sensitivity to antidiabetic drugs and being able to follow the pathogenesis of the disease and being suitable for routine treatment.

In experimental planning, it is very important to choose an animal model in terms of reliability, reproducibility of the results and guiding the researches by shedding light on the future. We recommend that the following items be considered while making this choice.

- A) The original features of the selected model, changing features depending on age and gender.
- B) Applications made depending on the characteristics of the animal must be stable throughout the experiment.
- C) Identified specific priorities should be well documented and reported. (Age, sex, reproductive history)
- D) In order to avoid problems that may occur during genetic studies, it should be ensured that it comes from a suitable breeding program by working with reliable suppliers.

Furthermore, animal models of disease have contributed to helping scientists and researchers better understand the mechanisms of other diseases in preclinical studies that allow screening of drugs and pharmaceutical agents, but their value in predicting the efficacy of treatment strategies in clinical trials remains controversial. Thus, further future studies are needed to explore the role of veterinary diseases in combination with pharmacological therapy. In addition, the number of animals and the discomfort to the animal should be kept to a minimum while designing the experiment. The results

of similar studies from different institutions should be carefully examined. Animal models designed with attention to all these have an important place in the final treatment of diabetes.

Therefore, the animal models that can be created while designing the study should be investigated very well; does it benefit the study, which model is chosen, statistically better results will be obtained, how to create a better animal model, will it have positive effects on human health at the end of the study, after getting answers to the questions, the study should be started.

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