Optimization of Glucose Loading Time and Alloxan Dosage for Inducing Stable Diabetes

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Abstract
Optimization of alloxan dosage is essential for inducing long-term and stable diabetes in experimental animals for diabetes testing. The purpose of this study is to determine the optimum of glucose loading time and alloxan dosage for inducing stable diabetes. Glucose loading times were evaluated at intervals of 30, 45, and 60 minutes following the administration of glibenclamide to male Wistar rats that had previously undergone fasting and received a glucose load of 1.35 g/kg.bw. Blood glucose levels were assessed at 0, 30, 60, 90, 120, and 180 minutes after glucose loading. The determination of the optimal glucose loading time was based on the AUC0–180 value calculated from blood glucose measurements using the trapezoidal formula. Intraperitoneal administration of alloxan at doses of 100, 120, 125, 135, and 150 mg/kg of body weight was conducted (n=6). Diabetes status was determined by assessing blood glucose levels on days 0, 7, 14, and 21, and the count of live rat, diabetic rat, and stable diabetic rat was recorded. The optimal timing for glucose loading in glucose tolerance testing (OGTT) with glibenclamide is 60 minutes after drug administration. Alloxan doses of 125, 135, and 150 mg/kg demonstrated consistent and stable diabetic outcomes, with the 125 mg/kg dose producing the highest number of stable diabetic rat. Consequently, the optimal timing for glucose loading is 60 minutes after drug administration, and the optimal alloxan dose for inducing stable diabetes is 125 mg/kg.bw.

INTRODUCTION
Diabetes mellitus (DM) is one of society's most prevalent metabolic disorders, distinguished by elevated blood glucose levels due to disruptions in insulin secretion, insulin action, or both (Ighodaro, 2018). Globally, nearly half a billion individuals (comprising 9.3% of adults aged 20-79) grapple with diabetes, and its prevalence shows a persistent upward trajectory. Consequently, the quest for and research on novel and improved diabetes treatment medications continues to intensify (Internation Diabetes Federation, 2019). The pursuit of new diabetes drugs involves conducting the oral glucose tolerance test (OGTT) and employing test animals induced with diabetogens. One aspect of OGTT results is influenced by drug or food intake before glucose loading in these test animals (Yoshioka et al., 1998). Preceding the OGTT test with the consumption of a low-carbohydrate diet or specific medications enhances glucose tolerance.
and tissue sensitivity to insulin. The commonly employed glucose preload times include 30, 45, and 60 minutes, resulting in varying OGTT outcomes (Mandlik et al., 2008; Chaimum-aom et al., 2017). Hence, it is essential to determine the optimal timing for glucose loading in the OGTT. Surprisingly, existing literature does not provide information on the optimal timing for glucose loading in the OGTT test.

The use of test animals such as induced rats is the easiest and most convenient method for screening new diabetes drugs. Therefore, it is crucial to determine the dosage of the drug used in diabetes induction to develop stable diabetes characteristics with minimal drug toxicity. One of the diabetogens is alloxan.

Alloxan is a commonly used diabetogen in test animals (Szkudelski, 2001; Malaisse et al., 2006). The dosage of alloxan to induce diabetes varies depending on the route of administration, the species of test animal, their age, and nutritional status. It has been reported that animals fasted overnight are more susceptible to alloxan (Szkudelski et al., 1998), while the presence of glucose when rats are induced with alloxan is known to protect pancreatic β-cells (Szkudelski, 2001). The range of alloxan diabetogen dosages is quite narrow, and even high doses can result in the death of test animals. This death is believed to be caused by the nephrotic toxicity of renal tubular cells at high doses of alloxan (Szkudelski, 2001). The intravenous dose of alloxan commonly given to rats is 65 mg/kg of body weight (Boylan et al., 1992). Meanwhile, the effective dose of intraperitoneal alloxan administration is 2-3 times the intravenous dose. Various literature sources have reported varying alloxan dosages ranging from 100-200 mg/kg of body weight (Ighodaro et al., 2017). The manifestation of diabetes conditions evaluated by blood glucose levels also varies from 150-750 mg/dL. These differences in glucose levels are influenced not only by the alloxan dosage but also by the induction period and the type of test animals used. Information about alloxan dosage optimization is limited, with one report by Ashok et al., (Ashok et al., 2007) on laboratory-maintained white rats at Shivaji University's Biochemistry Department in Kolhapur, India. The reported optimal dose of alloxan for inducing stable diabetes is 160 mg/kg of body weight. The scarcity of information regarding this optimal dose is regrettable, as it plays a crucial role in the success of diabetes drug testing.

This research was conducted to assess the optimal glucose loading time and the optimal dosage of alloxan for inducing stable and measurable diabetes. The optimal glucose loading time was determined by the lowest AUC value, while the optimal alloxan dosage for inducing stable diabetes was determined by the highest percentage of stable diabetic rats.

**METHOD**

The research design used in this study is a completely randomized design (CRD). The testing for the optimal glucose loading time was conducted at 30, 45, and 60 minutes after drug administration (glibenclamide 5 mg, Indofarma, Indonesia), with each treatment repeated three times. The observed parameters included blood glucose levels at 0, 30, 60, 90, 120, and 180 minutes.

Alloxan doses of 100, 120, 125, 135, and 150 mg/kg of body weight were used for testing the optimal stable diabetes dosage and were observed over 21 days. Blood glucose levels were measured on days 0, 7, 14, and 21. The parameters observed included blood glucose levels, the number of live mice, the number of diabetic mice, and the number of stable diabetic mice. Blood glucose testing was conducted using the OGTT method and measured using the GlukoDr. AutoTM Model AGM-400 from Korea.

Alloxan and glucose monohydrate were purchased from Sigma Chemicals, USA. Male albino rats (*Rattus norvegicus*) of the Wistar strain, weighing between 180-250 g and aged 3-4 months, were used. The rats were fed BR 2 diet and had access to water ad libitum. They were kept in an environment with a temperature range of 25-30 °C and humidity between 65-94%. The research was conducted at the Zoology Laboratory, UIN Mahmud Yunus Batusangkar, West Sumatra, Indonesia.

The optimal glucose loading time was determined through the Oral Glucose Tolerance Test (OGTT). Rats were fasted overnight (± 15 hours). The rats were divided into treatment groups (30, 45, and 60 minutes), with each treatment having three repetitions. Glucose was
administered after the administration of the drug (glibenclamide at 0.45 mg/kg of body weight). Just before glucose loading, blood glucose levels were measured (as T0), and blood glucose measurements continued at 30, 60, 120, and 180 minutes after glucose loading.

Diabetes was induced intraperitoneally with alloxan in rats that had been fasted overnight (± 15 hours). A total of 36 diabetic rats were divided into six dosage groups (0, 100, 120, 125, 135, and 150 mg/kg of body weight), each consisting of six rats. Alloxan was dissolved in a 0.9% NaCl solution before administration. Control animals (treatment 0) were only given a 0.9% NaCl solution intraperitoneally according to their body weight. Four days after alloxan induction, blood glucose checks were performed and considered as day 0. Blood glucose measurements continued on days 7, 14, and 21. Rats with blood glucose levels > 200 mg/dL were classified as diabetic. The number of live rats, diabetic rats, and stable diabetic rats were also recorded.

Blood glucose data at the optimal glucose loading time were analyzed, and the AUC0-180 value was calculated using the trapezium formula. The AUC0-180 values were subjected to tests for normality and homogeneity. If the data were homogeneous, a One-Way ANOVA test and Duncan's post hoc test were conducted. A significance level of P < 0.05 was considered statistically different. The data on the number of live mice, diabetic mice, and stable diabetic mice were calculated as percentages and analyzed descriptively.

Table 1. The effect of glucose loading time on the AUC0-180 values (n=3).

<table>
<thead>
<tr>
<th>Times</th>
<th>AUC0-180 Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 menit</td>
<td>11380 ± 780.05a</td>
</tr>
<tr>
<td>45 menit</td>
<td>11120 ± 921.29a</td>
</tr>
<tr>
<td>60 menit</td>
<td>9653.33 ± 260.88b</td>
</tr>
</tbody>
</table>

Numbers followed by the same letters in the same column are not significantly different in the DNMRT test at the 0.05 level.

The decrease in blood glucose can be attributed to the optimal absorption of the drug, allowing it to effectively reach the target tissues, ultimately aiding in the metabolism of the administered glucose in the rat’s body. This is substantiated by the observation of the lowest AUC value (Table 1). A lower AUC value corresponds to a greater reduction in blood glucose levels. On the other hand, a high AUC value indicates a higher concentration of circulating blood glucose systemically. This glucose loading time can serve as a benchmark for testing diabetes drug compounds derived from natural sources like plants, especially when glibenclamide is used as a control. Nevertheless,
recent research has also explored the optimal timing for glucose loading.

The optimal dosage of alloxan for inducing stable diabetes ranges from 125-150 mg/kg of body weight (Figure 1). This is evident from the stable blood glucose levels observed from day 7 to day 21 of observation. The blood glucose levels in stable diabetic rats induced with 125 mg/kg of alloxan ranged from 353 to 414.33 mg/dL. Meanwhile, at doses of 135 and 150 mg/kg of body weight, the blood glucose levels in diabetic rats ranged from 462 to 594.33 mg/dL.

The dose of 125 mg/kg of body weight of alloxan is capable of inducing diabetes and maintaining stability up to day 21, even when extended to 30 days. The same holds true for alloxan doses of 135 and 150 mg/kg of body weight. The stability of diabetes in rats induced with doses of 125, 135, and 150 mg/kg of body weight is attributed to alloxan’s ability to degenerate the pancreas through necrosis, leading to cell death (Elsner et al., 2002). Additionally, there is a deficiency in endogenous antioxidants, resulting in an abundance of reactive oxygen species (ROS) within the cells, exacerbating cell apoptosis. High ROS levels hinder the pancreatic cells from regenerating effectively. To counter ongoing cell apoptosis, exogenous antioxidants from external sources are needed to protect and aid in pancreas regeneration.

In Figure 1, it is also observed that the treatment with a dose of 100 mg/kg lacked the capability to induce diabetes, as indicated by the blood glucose levels on day 7, which remained normal and even approached the control mice that were not induced with alloxan. The dose of 120 mg/kg of body weight had the ability to induce diabetes on day 7, but it was unstable and returned to normal on day 14. The instability of diabetes in rats at the dose of 120 mg/kg of body weight was due to the fact that at this dose, its diabetogenic effect is low in degrading pancreatic beta cells compared to the pancreas’s inherent capacity to regenerate damaged cells. The body possesses mechanisms for regenerating the damaged pancreas through various processes (Cho et al., 2018), including the activation of specific genes and the differentiation of alpha cells into beta cells or the differentiation of acinar cells into Langerhans islets (Masuda et al., 2015). The faster the pancreas regenerates, the faster blood glucose levels return to normal because the pancreas and its produced hormones can regulate excess glucose.

Alloxan doses of 135 and 150 mg/kg of body weight can induce stable diabetes in rats, although in a very low percentage (16.67%) (Table 2). High doses of alloxan are toxic, not only to the pancreas but also to other organs such as the kidneys. Kidney damage is the primary reason for the high mortality of test animals.

![Figure 1](image-url)
during the induction of high-dose alloxan (Ashok et al., 2007; Ighodaro et al., 2017).

Table 2. Comparison of the effects of different doses of alloxan administration on the percentage of surviving rats, diabetic rats, and stable diabetic rats during a 21-day observation period (n=6)

<table>
<thead>
<tr>
<th>Treatments (mg/kg bw)</th>
<th>Surviving rats (n)</th>
<th>Diabetic rats (%)</th>
<th>Stable diabetic rats (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Dosage 100</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Dosage 120</td>
<td>5</td>
<td>83.33</td>
<td>0</td>
</tr>
<tr>
<td>Dosage 125</td>
<td>5</td>
<td>83.33</td>
<td>66.7</td>
</tr>
<tr>
<td>Dosage 135</td>
<td>2</td>
<td>33.33</td>
<td>33.33</td>
</tr>
<tr>
<td>Dosage 150</td>
<td>1</td>
<td>16.67</td>
<td>16.67</td>
</tr>
</tbody>
</table>

In Table 2, it is also evident that the dose of 125 mg/kg of body weight is the one with the highest success rate in inducing diabetes (83.33%) and achieving stable diabetes (66.70%). Alloxan doses of 135 and 150 mg/kg also can induce stable diabetes, but the number of rats that died was higher compared to those that survived or attained stable diabetes. High doses of alloxan have toxic effects on other tissues and organs in the body, not only pancreatic beta cells. This toxicity can cause severe adverse reactions and even death in some animals. The exact mechanism of alloxan toxicity is not fully understood, but is believed to involve the generation of reactive oxygen species (ROS) and oxidative stress, leading to cell damage and dysfunction (Delfita et al., 2021; Delfita et al., 2022).

The optimal dosage for inducing stable diabetes in test animals obtained in this study differs from the findings of Jörns et al. (Jörns et al., 1997) and Ashok et al. (Ashok et al., 2007), where the recommended doses for diabetes induction were 150 and 160 mg/kg of body weight, respectively. Additionally, a dose of 180 mg/kg of body weight was also recommended for inducing stable diabetes (Dewalkar & Masram, 2018). These variations in dosage are expected because numerous factors influence the effectiveness of alloxan as a diabetogen. Some of these factors include the age of the test animals, the duration of alloxan solution preparation before induction, the solvent used for alloxan, the temperature for dissolving alloxan, diet, and the route of alloxan administration. Alloxan dissolved in water can result in the formation of alloxanic acid, reducing or eliminating its diabetogenic properties (Lenzen & Munday, 1991). Younger rats are more resistant to alloxan compared to older ones, requiring higher doses (Jain & Arya, 2011). Alloxan is highly unstable at room temperature, affecting its diabetogenic properties, so the duration of induction plays a role. Diet or the presence of glucose affects alloxan's diabetogenic properties. The presence of glucose, through specific mechanisms, protects the pancreas from alloxan toxicity (Szkudelski, 2001). Hence the need to fast the rats overnight (at least 12 hours). In our study using Wistar rats, aged 3-4 months, alloxan was prepared just before induction, dissolved in 0.9% NaCl, and the rats were fasted overnight (± 15 hours). The rats were not given food for approximately 24 hours but were provided with a 5% glucose solution after 8 hours of induction.

The variability in the optimal dose found in various references for inducing stable diabetes highlights that predicting the exact optimal dose of alloxan as a diabetogen is challenging due to the numerous factors involved in determining its activity.

**CONCLUSION**

The optimal glucose loading time for testing antidiabetic activity is 60 minutes after drug administration. The optimal dose of alloxan for inducing stable diabetes is 125 mg/kg of body weight.

This study has limitations, including a limited number of observed parameters and a small sample size of tested rats. In the future, research with longer durations and more parameters, such as body weight, kidney and liver histology, is needed.
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REFERENCES


