Research article

Self-recovery in diabetic Sprague Dawley rats induced by intraperitoneal alloxan and streptozotocin

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ABSTRACT

Alloxan and streptozotocin are the most popular diabetogenic agents in assessing antidiabetic activity. Self-recovery, indicated by unstable hyperglycemia conditions in animals induced by those agents, becomes a significant disturbance to accurate examination. This study aimed to evaluate and reveal the self-recovery incidence in Sprague Dawley rats induced with alloxan and streptozotocin. Each dose of alloxan (120, 150, 180 mg/kg) and streptozotocin (40, 50, 60 mg/kg) was administered through intraperitoneal injection. The results showed that each dose of alloxan induced self-recovery incidence. In rats given streptozotocin, self-recovery only occurred at a dose of 40 mg/kg. The other higher doses of streptozotocin induced stable hyperglycemia. Furthermore, this study revealed two types of self-recovery, namely temporary recovery and end recovery. Temporary recovery occurred in rats given alloxan, during end recovery in alloxan and streptozotocin. The examination of insulin levels showed a significant reduction in the temporary recovery and stable diabetic rats compared to the end recovery rats. Besides, the bodyweight of rats was also affected by different incidences of self-recovery. This study recommends paying more attention to the possibility of self-recovery in obtaining animal models of diabetes, emphasizing the determination of suitable diabetogenic agents and proper doses to reduce self-recovery incidences. The finding of temporary recovery in rats receiving alloxan indicates that alloxan induced delayed diabetes in rats.

1. Introduction

Alloxan and streptozotocin have widely been used to produce animal models of diabetes because of their very cost-effective and expeditious technique [1,2]. The diabetogenic activity of alloxan was first reported by Dunn and McLetchie [3,4]. This agent-induced pancreatic beta cell necrosis is mediated by excess generation of free radicals in rabbits [5]. In the following years, alloxan is also used

to induce diabetes in rodents [2,6]. Meanwhile, streptozotocin as a diabetogenic agent was first announced by Rakieten and colleagues [7]. Streptozotocin causes DNA alkylation of Langerhans beta cells [8]. Streptozotocin induces diabetes in pigs, hamsters, monkeys, dogs, and rodents such as rats and mice [6,9,10].

Rodents have become the most widely used animal model in biomedical sciences. Their advantages are that they are easy to handle and have shorter generation intervals to complement the required experimental animals [11,12]. Also, the principles of animal research guidelines utilize the lowest level of animal species available [11]. The physiology of rodents is closer to that of humans than non-mammalian animal models [6,13]. They are also more convenient and less expensive, explaining why most experiments are performed on rodents, including diabetes.

The effectiveness of alloxan in inducing diabetes, especially in rodents, has recently become a topic of discussion. Several researchers argue that alloxan is neither optimal nor ideal as a diabetogenic agent regarding its spontaneous recovery [14,15]. In the recovery case, mild diabetes produced by alloxan recuperated from their condition. Thus high blood glucose levels returned to normal levels [14,15].

A previous study becomes a reference for several researchers who argue that alloxan is not ideal. In detail, however, spontaneous recovery in the study occurred at single or multiple low doses of alloxan, from 30 to 40 mg/kg [16]. Nowadays, the dose of alloxan to induce diabetes in rats is higher than that used in the study, which varies from 90 to 200 mg/kg, and the most widely used is 150 mg/kg [2]. Compared to alloxan, streptozotocin is preferred as a diabetogenic agent [2]. A total of 57.9% of studies used streptozotocin, while 30.3% used alloxan, and others used compounds such as monosodium glutamate, dithizone, glucose, and fructose [2,11].

Previous studies have reported self-recovery in diabetes rats induced using intravenous injection of low dose streptozotocin at 40 mg/kg [17,18]. Technically, the dose of 40 mg/kg streptozotocin or lower is still used in intraperitoneal induction of diabetes [19]. There is no information regarding self-recovery in Sprague Dawley rats induced intraperitoneally using this agent. The present study aimed to directly compare self-recovery in Sprague Dawley rats induced diabetes through intraperitoneal injection with alloxan and streptozotocin.

2. Materials and method

2.1. Experimental animals

Thirty-five male Sprague Dawley rats weighing 230–250 g at eight weeks of age were used in this study. The animals were purchased from The Experimental Animal Facility of The National Agency of Drugs and Food Control (BPOM), the Republic of Indonesia. All animals received commercial standard rat food and water ad libitum and were caged under controlled laboratory conditions. The animals were acclimatized for two weeks before the experiments. All experimental procedures and use of animals were approved by the Animal Ethics Committee of The Veterinary Teaching Hospital, Faculty of Veterinary Medicine, IPB University (No. 29–2016 ACUC RSHP FKH-IPB) and were following the recommendations of the proper care and use of laboratory animals.

2.2. Diabetes induction materials

Diabetes in Sprague Dawley rats was induced chemically by diabetogenic agents streptozotocin and alloxan monohydrate. Each rat received a diabetogenic agent with a single dose. Both streptozotocin and alloxan were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

2.3. Diabetogenic agent induction

Rats were randomly divided into seven groups, the three groups received three doses of streptozotocin, and the other three received three doses of alloxan. The remaining group received blank injections as a control group (without streptozotocin and alloxan).

Streptozotocin was freshly prepared in a 0.1 M citrate buffer pH 4.5 immediately before injection. Streptozotocin was injected intraperitoneally in the fasted rats at a 40, 50, and 60 mg/kg single dose level. Sucrose solution (10%) was administered for 24 h to avoid mortality due to hypoglycemic shock after the induction of streptozotocin-induced rats [20].

Alloxan solution was prepared by dissolving in normal saline and used immediately. Fasted rats were intraperitoneally injected with an alloxan monohydrate solution at doses of 120, 150, or 180 mg/kg. Sucrose solution (10%) was not administrated in alloxan-induced rats related to prevent the protective mechanism against alloxan-induced β cell damage [21,22].

2.4. Fasting blood glucose level and body weight

The fasting blood glucose (FBG) level and bodyweight were measured weekly for 28 days. Blood glucose was measured using a glucometer Accu-Chek® Active (Roche Diagnostics GmbH, Mannheim, Germany) with a tail bleed.

2.5. Self-recovery assessment

Self-recovery was categorized for rats that had normal blood glucose levels (FBG <151 mg/dl) after hyperglycemia was achieved (FBG >150 mg/dl) by induction of alloxan or streptozotocin.

2.6. Insulin serum

Approximately 0.5–1 mL of blood was collected from the rats' tails, then transferred to red top vacutainer tubes without additive and centrifuged to collect serum at 1300 RCF for 10 min using Micro spin 12 high-speed mini-centrifuge SIA BIOSAN, Latvia. Serum samples were stored at $-20\,°C$ until analysis. Serum insulin levels were determined using The Rat Insulin ELISA Kit, catalog# 90,010 (Crystal Chem Inc), according to the manufacturer's instructions.

2.7. Statistical analysis

The incidence of self-recovery in alloxan and streptozotocin-induced diabetic rats were compared using Fisher's exact test ($p <$ 0.05). Insulin level and body weight of rats were tested for normality (Shapiro-Wilk) and homogeneity (Levene), then compared with ANOVA and followed by Duncan's Multiple Range Test (DMRT) ($p < 0.05$ or $p < 0.01$).

3. Results

3.1. Self-recovery in alloxan and streptozotocin-induced diabetic rats

Self-recovery in rats induced with alloxan and streptozotocin is presented in Table 1. Seven Sprague Dawley rats exhibited self-recovery, 4 rats from alloxan, and 3 rats from streptozotocin. Self-recovery developed at all tested doses of alloxan (120, 150, and 180 mg/kg). Meanwhile, in streptozotocin-induced diabetic rats, it only occurred at the lowest dose (40 mg/kg).

The statistical evaluation showed no significant difference in the self-recovery proportion among the alloxan dose groups ($p > 0.05$). Meanwhile, significant differences in self-recovery were found in rats receiving various doses of streptozotocin ($p < 0.05$). Besides, the proportion of self-recovery was also significantly different between alloxan and streptozotocin-induced diabetic rats ($p < 0.05$).

3.2. Identification of self-recovery types

Two types of self-recovery were distinguished in this study, namely temporary recovery and end recovery, as depicted in Fig. 1. Among the 4 self-recovery rats induced by alloxan, 3 rats had temporary recovery and 1 rat had end-recovery. Meanwhile, 3 rats induced with streptozotocin showed end-recovery. There were no temporary recovery rats in streptozotocin-induced diabetic rats.

All temporary recoveries were identified in week-2 after the rats were induced. The high blood glucose of the rats returned to normal levels at that time, but then the normal blood glucose levels continuously increased from week-3 to week-4. Meanwhile, end-recovery was found at various times between week-2 and week-4.

Self-recovery occurred not only in rats with intermediate blood glucose levels but also in rats with severe blood glucose levels. Five rats with self-recovery had a severe level of blood glucose.

3.3. The effect of self-recovery to insulin levels

The examination of insulin reduction is presented in Table 2. Significant insulin reduction occurred in temporary recovery rats and was similar to the reduction of stable diabetic rats. Meanwhile, end recovery rats had the smallest insulin reduction compared to the stable diabetic and temporary recovery rats ($p < 0.05$).

Table 1
Self-recovery in alloxan and streptozotocin-induced diabetic rats.

Diabetogenic Agents	Doses (mg/kg)	Induced animals	Die & ND	Self-recovery (n)		p-Value	
				Present	Absent		
None	0	5	0	–	–	–	
Alloxan	120	5	3	2	0		
	150	5	4	1	0	1.000	
	180	5	4	1	0		
Total				4	0		0.025
Streptozotocin	40	5	1	3	1		
	50	5	1	0	4	0.024	
	60	5	2	0	3		
Total				3	8		

Fisher's exact test ($p < 0.05$). In analyses, doses are grouped into low doses (Alloxan 120 and Streptozotocin 40 mg/kg) and high doses (Alloxan 150 dan 180 mg/kg and Streptozotocin 50 dan 60 mg/kg). Die & ND (Non-Diabetes): animals died or did not show increased in blood glucose until 7 days after induction.
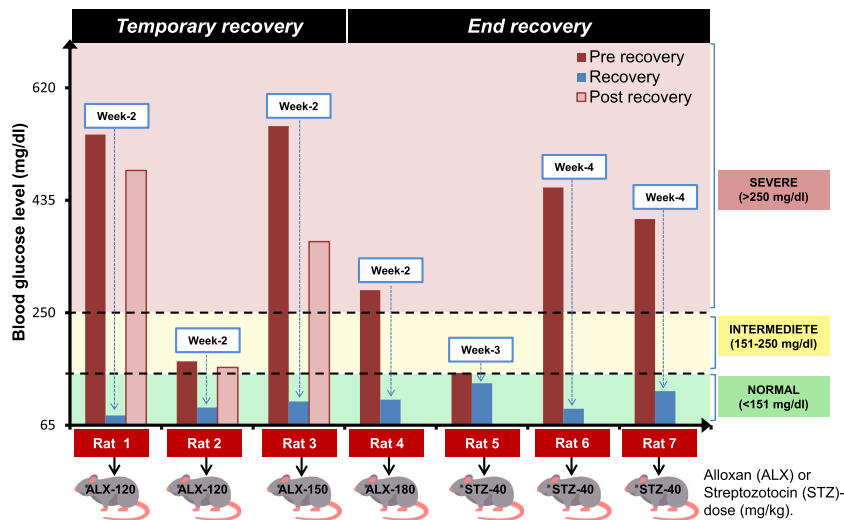
Fig. 1. Temporary recovery and end recovery in alloxan or streptozotocin-induced diabetic rats. The white box above the bar indicates the identified self-recovery time.

Table 2
The reduction of insulin level.

Group	Animal (n)	Insulin level (ng/ml)		Change of insulin level (%)
		Pre	Post	
Non-diabetes	3	0.383 ± 0.072	0.343 ± 0.177	24.4 ± 4.114[a]
Stable diabetes	3	0.469 ± 0.061	0.069 ± 0.012	85.3 ± 0.574[c]
End recovery-STZ	3	0.461 ± 0.212	0.116 ± 0.036	73.7 ± 4.097[b]
End recovery-ALX	1	0.367	0.098	73.30
Temporary recovery-ALX	3	0.465 ± 0.115	0.081 ± 0.013	82.7 ± 3.148[c]

Data were normally distributed and homogeneous. This data was then analyzed statistically using ANOVA followed by Duncan's Multiple Range Test (DMRT). Different letters (a, b, and c) showed a significant difference $p < 0.05$. There was only 1 individual in the End Recovery-ALX group and excluded during statistical tests.

3.4. The effect of self-recovery on bodyweight

The bodyweight examination of rats is presented in Fig. 2. The effect of different self-recovery on the bodyweight of rats occurred at week 4 after induction. The end-recovery rats had significantly higher body weight compared to the stable diabetic rats ($p < 0.05$). Meanwhile, there were no significant differences in the bodyweight of temporary recovery rats compared to the diabetic rats.
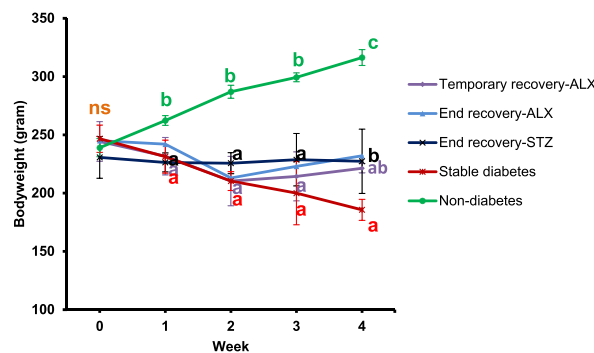

Fig. 2. Changes of body weight. The same letter in the same week means not significantly different ($p < 0.05$) using Duncan's Multiple Range Test (DMRT).

4. Discussion

A significant rise in blood glucose is pivotal in establishing animal models of diabetes. High blood glucose levels are used to assess various antidiabetic candidates. Meanwhile, the present study showed that several rats induced with alloxan and streptozotocin could restore high blood glucose to normal levels, called self-recovery. Self-recovery is the term used to define the high blood glucose levels naturally returning to normal levels, not as a result of medication or therapy. Every diabetic rat induced by alloxan exhibited self-recovery. This result occurred at every dose of alloxan tested (120, 150, and 180 mg/kg). In streptozotocin-induced rats, self-recovery occurred at the low dose (40 mg/kg).

A previous study reported that self-recovery occurred in Wistar and Sprague Dawley rats induced through intravenous streptozotocin at 40 mg/kg [17,18]. It differs from the present study, which used an intraperitoneal induction route. Animal strains and route of administration could affect the reproducibility of diabetic animal models [9,11,23]. However, those studies and the current study showed that self-recovery occurred at a similar dose of streptozotocin, even though the strain of rats and route of administration differed.

This study suggests that the incidence of self-recovery is an essential indicator in determining the optimal dose of diabetogenic agents, in addition to other factors. Based on the self-recovery incidence, a 40 mg/kg of streptozotocin is inappropriate for producing diabetes. Thus, using a single dose of 40 mg/kg streptozotocin to induce diabetes in rats needs to be reconsidered. At a low dose (20–40 mg/kg), streptozotocin is perhaps effective at multiple administrations depending on the species and strain [24]. Contrary to the current study, a previous report assessed that 40 mg/kg of streptozotocin was the optimal dose to produce diabetic Wistar rats since mortality decreased and diabetes incidence increased [25].

Furthermore, the present study identified two different types of self-recovery occurring in alloxan and streptozotocin-induced diabetic rats, namely temporary recovery and end recovery (Fig. 2). Temporary recovery was indicated by high blood glucose levels after induction recovering to normal level (blood glucose levels <151 mg/dl), but after that, the blood glucose levels returned to intermediate level (blood glucose levels 151–250 mg/dl) or severe level (blood glucose levels 151–250 mg/dl) on the following week's monitoring. Meanwhile, end recovery refers to permanent recovery, not accompanied by a return of blood glucose level to hyperglycemia (intermediate or severe levels) after recovery. Self-recovery that occurs at the end of the observation is also classified as end recovery.

Two types of self-recovery have significantly different effects on insulin reduction (Table 2). The insulin decrement in end recovery rats was significantly smaller than in the decrease in temporary recovery rats. The lower decrease in insulin levels is expected to lead to the incidence of end recovery in rats receiving low doses of streptozotocin. According to the study of Ar'Rajab and Ahren, the insulin secretion in rats induced with low doses of the diabetogenic agent was less than that of non-diabetic rats [18]. However, the remaining insulin was still sufficient in returning the high blood glucose level to normoglycemic. Meanwhile, the reduction of insulin was similar between temporary recovery rats and stable diabetic rats (Table 2). This result supported the existence of the temporary recovery.

The interaction between natural endogenous antioxidants produced by the body and radical oxygen species generated by diabetogenic agents could contribute to end-recovery in rats induced by the low dose of agents. The low doses of streptozotocin generate low radical oxygen species (ROS) [26]. Endogenous antioxidants can counteract ROS [23,27]. This condition provides an opportunity for end recovery because a small amount of ROS could be well attenuated by endogenous antioxidants. Thus, increased severe damage to beta cells in the pancreas due to ROS can be inhibited.

In contrast, the abundance of ROS in rats that received high doses of agents led to increasingly intricate recovery and thus resulting in more stable hyperglycemia. On the other hand, this study found an end-recovery incidence in a rat receiving a high dose of alloxan (180 mg/kg). A study reported that some individuals have high levels of antioxidants correlated with their gene overexpression, making them resistant to the agent [28]. The limitation of this study, the number of antioxidants was not measured to support it. The opportunity of end recovery in animals receiving high doses of agents may be higher in alloxan-induced rats than the streptozotocin. This surmise is related to the difference in the primary mechanism of the two agents in inducing diabetes. Alloxan induced diabetes by increased oxidative stress as the primary mechanism, while streptozotocin by alkylation of DNA [1].

The evaluation of body weight also supported two types of self-recovery (Fig. 2). End recovery rats' body weight was significantly higher than that of the stable diabetic rats in week 4 after induction. Meanwhile, the bodyweight of temporary recovery rats was significantly similar to that of stable diabetic rats. The bodyweight of diabetic rats is associated with hyperglycemia [29]. In end recovery rats, hyperglycemia returned rapidly to the normal level, thus, the bodyweight loss of rats was slighter.

Unlike end recovery, temporary recovery has never been reported. Several studies showed that alloxan produced unstable diabetes conditions [14,15,30]. The blood glucose level returned to normal in 10 days [30]. This case is similar to the end recovery because there was no information regarding hyperglycemia after recovery. The application of alloxan as a diabetogenic agent is controversial. Several studies reported that alloxan is a non-ideal diabetogenic agent [14,15]. However, many studies have successfully used this agent [31,32]. Various antidiabetic candidates were assessed using alloxan-induced diabetic rats [29,33–35]. Interestingly, through continuous observations after self-recovery incidence at week 2 in alloxan-induced diabetic rats, their blood glucose levels rose continually in the following weeks (Fig. 2), so temporary recovery was identified. Insulin level reduction and body weight had significant similarities between temporary recovery and stable diabetic rats at the end of the observation, thus supporting this case (Table 2 dan Fig. 2).

Temporary recovery in alloxan-induced rats could correlate with the multiphasic blood glucose response. This response is that blood glucose levels fluctuate before reaching stable hyperglycemia as a reaction to alloxan induction [2]. Multiphasic blood glucose level persists for the first few hours up to 48 h after induction [1,2]. However, it is suspected that multiphasic blood glucose response could occur for a longer time, making it responsible for the incidence of temporary recovery.

Temporary recovery indicated that alloxan could produce diabetic rats, but alloxan generated delayed diabetes. In this study, alloxan-induced diabetic rats had unstable blood glucose levels in the first 2 weeks. However, they were followed by continuous hyperglycemia in the following weeks, changes in body weight, and a significant decrease in insulin level. Temporary recovery could be one of the answers to studies that have successfully used alloxan to induce diabetes, while others failed.

5. Conclusions

Self-recovery in Sprague Dawley rats induced by alloxan occurred at each dose tested (120, 150, and 180 mg/kg), while self-recovery in Sprague Dawley rats induced with streptozotocin only occurred at a dose of 40 mg/kg. Two types of self-recovery were identified, namely temporary recovery and end recovery. Temporary recovery was found in alloxan-induced diabetes, and the end recovery was in alloxan and streptozotocin-induced diabetes.

Author contribution statement

Indah Fajarwati; Tutik Wresdiyati: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.
Dedy Duryadi Solihin: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.
Irmanida Batubara: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Data availability statement

Data will be made available on request.

Additional information

No additional information is available for this paper.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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