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## **Optimization of Alloxan Dose is Essential to Induce Stable Diabetes for Prolonged Period**

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**Abstract:** Alloxan dose for experimental induction of diabetes in rats has to be optimized. Lower doses can result in auto-reversion to normal state without medical help, while higher doses cause toxicity and loss of experimental animals. Induction of stable diabetes for prolonged period is needed for studies on various parameters in diabetes. Alloxan dose of 120, 140, 160 and 180 mg kg<sup>-1</sup> body weight was given intraperitoneally to experimental rats in groups of 6 animals for each dose. The blood glucose, urine glucose, liver glycogen level and variation in body weight were studied. Alloxan toxicity to the kidney was evaluated histologically. OGTT (Oral Glucose Tolerance Test) was performed to determine the correct diabetic status of the animals. Lower doses up to 140 mg kg<sup>-1</sup> body weight induce diabetes, however animals revert back to normal blood glucose values in a week. Alloxan dose of 160 mg kg<sup>-1</sup> body weight can demonstrate stable diabetes and animals survive for months. While animals treated with 180 mg kg<sup>-1</sup> body weight show severe diabetes, high mortality rate and damage to kidneys. A dose of 160 mg kg<sup>-1</sup> body weight is suitable for induction of stable diabetes.

**Key words:** Alloxan, stable diabetes induction, alloxan toxicity, experimental diabetes

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### **INTRODUCTION**

The incidence of diabetes mellitus is increasing in the world and is assuming epidemic proportion. Search for newer and better drugs to treat the disease therefore, is also on the rise. The use of experimentally induced diabetes in rats remains as one of the easiest and convenient methods for screening of new drugs. Hence it is vital to establish a dosage of drugs used in induction of experimental diabetes so as to develop stable diabetic characteristics with minimal drug toxicity.

Alloxan is routinely used to induce diabetes in experimental animals ever since its ability to induce diabetes was reported (Dunn *et al.*, 1943). The dose of alloxan required for inducing diabetes depends on the animal species, route of administration and nutritional status. It is reported that animals kept on overnight fast are more susceptible to alloxan (Kastumata *et al.*, 1992; Szkudelski *et al.*, 1998), while glucose is known to protect the beta cells (Bansal *et al.*, 1980; Szkudelski, 2001). High glucose level prevents the superoxide derivative, which cause the cell damage (Martens *et al.*, 2005). It has been recognized that the range of the diabetogenic dose of alloxan is quite narrow and even light overdosing may be generally toxic causing the loss of many animals (Szkudelski, 2001). This loss is suggested to be due to kidney tubular cell necrotic toxicity at high doses of alloxan (Lenzen *et al.*, 1996). Alloxan rapidly and selectively accumulates in pancreatic beta cells (Gorus *et al.*, 1982; Elsner *et al.*, 2006) and is known to induce DNA strand breaks in isolated rat pancreatic islets (Okamoto, 2005). The most frequently used intravenous dose of this drug to induce diabetes in rats is 65 mg kg<sup>-1</sup> body weight. (Gruppuso *et al.*, 1990; Boylan *et al.*, 1992). When alloxan is given intraperitoneally or subcutaneously its effective dose must be 2-3 times higher. A survey of literature revealed different workers using

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doses of alloxan varying from 80-200 mg kg<sup>-1</sup> of body weight (Venkatesh *et al.*, 2003; Viridi *et al.*, 2003; Ananthan *et al.*, 2003; Nagappa *et al.*, 2003; Sabu and Kuttan, 2002; Gosh *et al.*, 2003; Arun and Nalini, 2002). The manifestation of diabetes state as evaluated by the blood glucose levels also therefore, varies from 150-750 mg dL<sup>-1</sup>. Differences also exist in the experimental time periods.

The present study was undertaken to evaluate the optimal dose of alloxan that can induce diabetes mellitus in stable and measurable manner. We have found that while lower doses can manifest as increased blood glucose levels the animals are able to revert back to their normal status without any medicinal help. On the contrary, higher doses induce severe toxicity resulting in reduction in survival rate beyond 20 days that hampers longitudinal experiments.

## MATERIALS AND METHODS

Alloxan and amyloglucosidase were purchased from Sigma Chemicals, USA. All other chemicals used for the study were of AR grade and purchased locally. Male albino rats weighing between 150-250 g were used. The rats were fed with standard rat diet purchased from Amruth India. Animals were provided with food and water *ad libitum* and maintained at 25-28°C. All the studies were carried out in the Department of Biochemistry of Shivaji University, Kolhapur.

Glucose was measured by the glucose oxidase method of Llyod and Whelan (1969) and also by using glucose oxidase kits purchased from Qualigens, India. Glucose for Oral Glucose Tolerance Test (OGTT) was measured using ACCU-CHECK purchased from Roche India and results verified using appropriate controls.

Each group in the study consisted of six rats. Diabetes was induced by injecting alloxan at dose levels 120, 140, 160 and 180 mg kg<sup>-1</sup> of body weight intra-peritoneally to overnight fasted animals. It was injected in single dose in total volume of 0.5 mL saline prepared freshly. Control animals were injected with the vehicle of 0.5 mL saline.

Liver glycogen was determined using method of Roe *et al.* (1961). Tissue was treated with 10% TCA, pH adjusted and glucose measured after amyloglucosidase treatment using glucose oxidase/peroxidase.

Oral glucose tolerance test was administered by feeding rats with 3 mg glucose g<sup>-1</sup> of body weight. Blood was withdrawn from tail vein at defined time intervals and blood glucose measured using Accu-check.

Overnight urine was collected in clean container and used for glucose estimation. For measurement of urine glucose the sample was deproteinised using 10% Na-tungstate and H<sub>2</sub>SO<sub>4</sub> and supernatant was used for glucose estimation using O-toluidine method.

After sacrificing the animals by cervical dislocation, kidneys were removed and immediately placed in 10% formaldehyde. The kidney tissue was embedded in paraffin and sectioned to get 5 µm sections with microtone for histological studies. Sections were stained with hematoxylin-eosin stain and observed at 60X

Statistical analysis was done by using ANOVA and Student's t-test. Data expressed as ±SD. Statistical significance was defined as p<0.05.

## RESULTS AND DISCUSSION

The effect of alloxan on blood glucose as depicted in Fig. 1 shows the efficacy of alloxan in induction of diabetes. Dosage of 180 mg kg<sup>-1</sup> of body weight appears to cause extensive damage of beta cells, in that the blood glucose levels goes above 630±10 mg dL<sup>-1</sup>. A dosage of around 140 mg kg<sup>-1</sup> of bodyweight that is commonly used in induction of diabetes can be observed to show an elevation of blood glucose to around 232.5±4.5 mg dL<sup>-1</sup> that is considered as diabetic by normal standards.

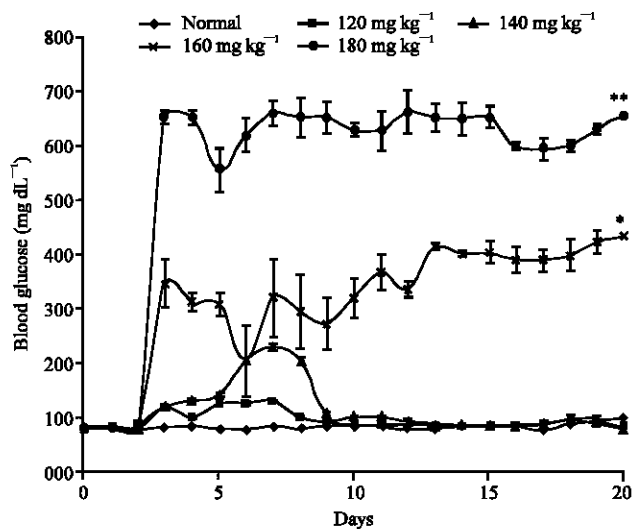


Fig. 1: Effect of increasing alloxan dose on blood glucose level with time. Animals were kept on an overnight fast of 12 h and blood glucose level recorded. On 20th day \* $p < 0.003$  and \*\* $p < 0.0002$ , when compared with normal animals

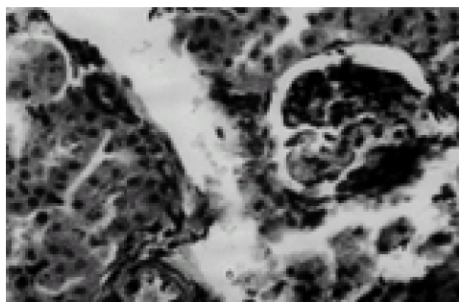


Fig. 2: Effect of alloxan at  $180 \text{ mg kg}^{-1}$  of body weight on rat kidney. White arrow indicates shrinkage in glomeruli and black arrow shows presence of phagocytes in tubules. (Magnification 60X)

However it should be noted that these values are observed only for period of 4-5 days. At  $120 \text{ mg kg}^{-1}$  body weight we observe similar elevation though towards lesser extent of  $127 \pm 5 \text{ mg dL}^{-1}$ . In both the doses the values are seen to normalize by 9 days. The dosage of  $160 \text{ mg kg}^{-1}$  of body weight however appears to maintain a blood glucose of  $300\text{-}400 \text{ mg dL}^{-1}$  throughout the experimental period.

The toxicity of alloxan was studied by observing the histological changes in the kidney. There was no damage observed at doses below  $140 \text{ mg kg}^{-1}$ . At  $160 \text{ mg kg}^{-1}$  the kidney shows stress however no necrotic damage was evident. Figure 2 however, demonstrates the damage occurring at  $180 \text{ mg kg}^{-1}$  alloxan dose. Shrinkage of glomeruli and presence of phagocytes in tubules can be observed.

Severe polyuria was observed, as an average 20 mL of urine was voided overnight in fasted animals treated with  $180 \text{ mg}$  alloxan  $\text{kg}^{-1}$  of body weight as against 10 mL for  $160 \text{ mg kg}^{-1}$  of body weight rat group and 5-7 mL for  $120 \text{ mg}$  and  $140 \text{ mg kg}^{-1}$  of body weight rat group.

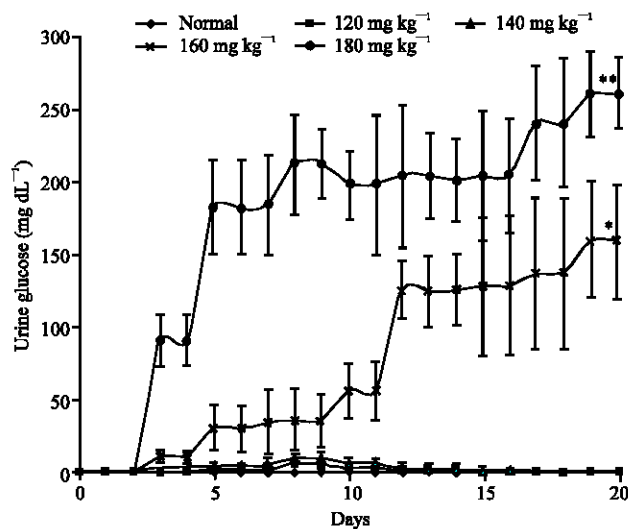


Fig. 3: Effect of alloxan on urine glucose level. Overnight urine was collected and total glucose content measured. \* $p < 0.003$  and \*\* $p < 0.0005$  vs. healthy animals

Table 1: Variation in body weight of experimental animals with increasing alloxan dose

Day	Normal	120 mg kg <sup>-1</sup>	140 mg kg <sup>-1</sup>	160 mg kg <sup>-1</sup>	180 mg kg <sup>-1</sup>
0	223.33±11.54	225.00±08.66	230.00±10.00	226.66±11.54	226.66±5.770
3	223.33±11.54	225.00±08.66	230.00±10.00	226.66±11.54	226.66±5.770
6	224.00±12.16	223.33±05.77	230.00±10.00	219.00±07.93	211.66±10.40
9	225.00±13.22	226.66±05.77	225.66±04.04	210.00±10.00	195.00±05.00
12	225.00±13.22	228.33±07.63	227.33±04.61	203.33±20.81	190.00±10.00
15	226.66±15.27	230.00±10.00	227.33±04.61	180.00±10.00	161.00±11.53
18	226.66±15.27	230.00±10.00	227.33±04.61	180.00±10.0*	143.33±12.58**

\* $p < 0.003$  for 160 mg kg<sup>-1</sup> and \*\* $p < 0.001$  for 180 mg kg<sup>-1</sup> groups when compared with healthy animals

The pattern of urine glucose variation follows the similar trend as the blood glucose levels in Fig. 1. Although blood glucose values increases to about 232.0±4.5 mg dL<sup>-1</sup> during 4-8 days in case of animals treated with 140 mg kg<sup>-1</sup> of body weight a similar increase in the urine glucose was not observed (Fig. 3).

Again, weight loss is rapid in the case of animals treated with 180 mg of alloxan kg<sup>-1</sup> of body weight. It is also to be observed that while animals treated with alloxan dose of 120 and 140 mg kg<sup>-1</sup> of body weight show reduction in weight initially, by 15th day they regain the lost weight and are comparable to the normal animals (Table 1).

Oral glucose tolerance test gives a better estimate of the diabetic status. In Fig. 4 both animals with 160 and 180 mg kg<sup>-1</sup> of body weight alloxan dose show a clear diabetic pattern of glucose tolerance. Animals on 140 mg kg<sup>-1</sup> of body weight of alloxan dose shows an initial shoot up of about 231.66±32 mg dL<sup>-1</sup>, but reverts to normal by 2 h. Even at 120 mg kg<sup>-1</sup> of body weight of alloxan dose which otherwise shows all parameters comparable to normal, demonstrate a small increase to about 180 mg dL<sup>-1</sup> as compared to normal value of 165.33±15.82 mg dL<sup>-1</sup>. However such an increase is not observed after about 40 days (data not shown). Although the results are presented after 20 days of experimental induction of diabetes, mainly owing to the mortality of alloxan at 180 mg kg<sup>-1</sup>, groups of animals were observed for over 3 months time period. Animals at 160 mg kg<sup>-1</sup> of alloxan continue showing blood glucose value of around 400 mg dL<sup>-1</sup> for 3 months.

A similar pattern is observed with respect to liver glycogen as seen in Table 2. Liver glycogen level is used as a parameter to study the status of carbohydrate metabolism. Rat liver slices *in vitro* (Baker *et al.*, 1952), demonstrated an almost complete suppression of the incorporation of labeled

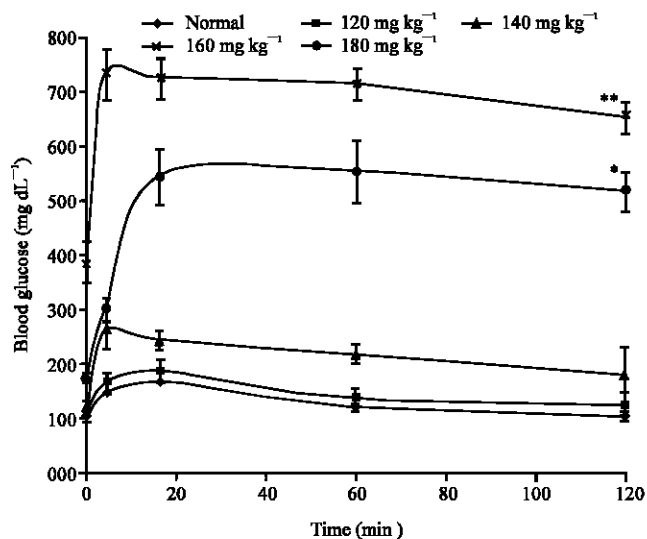


Fig. 4: Oral glucose tolerance test was carried out by administering glucose load of 3 mg g<sup>-1</sup> body weight to overnight fasting animals over a time period of two hours. On 20th day \*p<0.002 and \*\*p<0.0004, when compared with normal

Table 2: Liver glycogen levels of animals treated with varying dosage of alloxan. Liver glycogen was determined after 20 days.

Group	Glycogen levels in mg g <sup>-1</sup> of tissue
Normal	1.823±0.17
120 mg kg <sup>-1</sup>	1.400±0.26
140 mg kg <sup>-1</sup>	1.233±0.25
160 mg kg <sup>-1</sup>	0.600±0.05*
180 mg kg <sup>-1</sup>	0.453±0.12**

\*\*p-values at the 20th day of the 160 and 180 mg kg<sup>-1</sup> treated groups are less than 0.007\* and 0.0001 vs. healthy animals

glucose into the liver glycogen of alloxan treated diabetic rats. Alloxan induced diabetes results in a loss of liver capability to activate glycogen synthetase *in vitro* (Gold, 1970). Decrease in glycogen level appears to be dose dependent. Liver glycogen reduces to almost 65-75% in animals treated with 160 and 180 mg kg<sup>-1</sup> of body weight of alloxan dose, as compared to 25-28% decrease in animals treated with 120 and 140 mg kg<sup>-1</sup> of body weight of alloxan dose.

We have further observed manifestation of cataract after 15 days at 180 mg kg<sup>-1</sup> dose and in between 18-20 days in case of 160 mg kg<sup>-1</sup> alloxan dose. There was no development of cataract at lower doses.

Numerous studies are being carried out all over the world to gain insights into the anti-diabetic activity of different compounds as well as studying various parameters in diabetes. Alloxan is routinely used intraperitoneally to induce diabetes in experimental animals and constitutes a simple tool for testing the anti-diabetic potential or changes in analytes in the diabetic state. However, our literature review revealed usage of alloxan varies from 80 mg to even i.p., 200 mg kg<sup>-1</sup> of body weight. In the present study we have found that alloxan given at a dose of 160 mg kg<sup>-1</sup> of body weight is suitable to induce stable diabetes with minimal alloxan toxicity. It is possible to test for new drugs on diabetes within a time span of over two months during which the animals survive with frank diabetes. Many *in vivo* experimentation needs this time span to establish the validity of a new drug.

It was realized that alloxan dose at 140 mg kg<sup>-1</sup> body weight and below induced a temporary diabetic state and animals were able to revert back to normal values by 10 days. A similar report of recovery of endocrine function on administration of 120 mg kg<sup>-1</sup> body weight by 12 days was suggested to be due regeneration and neogenesis of pancreatic beta cells (Haro-Hernandez, 2003). Likewise a dosage below 150 mg kg<sup>-1</sup> body weight has been shown to be insufficient to induce diabetes earlier (Katsumata *et al.*, 1992, 1993). The values of liver glycogen as well as the body weight does not show appreciable changes at 120 and 140 mg kg<sup>-1</sup> of alloxan dose. We have observed that at lower dose of alloxan the animals do exhibit a changed pattern in the OGTT test however the blood glucose levels (fasting) does not reflect this change. Alloxan may be inducing changes, however over a time span at low doses the animals appear to recover back even without medical help.

Especially a dose of 140 mg kg<sup>-1</sup> body weight can be very misleading as blood glucose level increase to an accepted diabetic state by about 5th day of injection, however, this value reverts back to normal by the 9th-10th day. Hence experiments carried out with potential drugs in this time period may be wrongly interpreted as compounds having good anti-diabetic potential. We have noted antidiabetic potential attributed to new drugs even at 120 mg kg<sup>-1</sup> of alloxan dose that does not induce stable measurable diabetes.

Further, we have noted that a dosage at 180 mg kg<sup>-1</sup> body weight and above can induce diabetes, however, the extent of hyperglycemia is extreme. The animals show heavy polyuria and polydypsia and severe toxicity as more than 50% of the animals are lost. It is very likely that the toxicity may be due to severe damage to pancreatic islets in addition to loss of renal function. In this case it is difficult to plan and conduct longitudinal experiments over 20 days and interpretation of results is ambiguous. Hence we conclude that for experimental induction of diabetes alloxan dose of 160 mg kg<sup>-1</sup> body weight given intraperitoneally in single dose to fasting rats is best suited. The animals show frank diabetes, are able to survive for months in the diabetic state, develop secondary complications such as cataract and most important, do not revert back to normalcy without medical help. It is therefore very vital to use the correct alloxan dose to fasting animals as conclusions drawn on diabetic studies and new anti-diabetic drugs can lead to erroneous conclusions.

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