

EXPERIMENTAL PHARMACOLOGICAL MODEL OF DIABETES INDUCTION WITH ALOXAN IN RAT

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Abstract

An experimental pharmacological model of diabetes induced with aloxan in white, male and female Wistar rats was designed. A collectivity of 312 animals was used, with an initial mean weight of 225.1 ± 31.48 g-bw and basal mean glycaemia of 103.0 ± 34.22 mg/dL. Diabetes was induced by administering intraperitoneally a dose of 130 mg/kg-bw aloxan. Experimental results showed that, in the tested collectivity, 66.34% of animals became diabetic, with a mean glycaemic value of 450.4 ± 129.7 mg/dL. For the diabetic group, decreases in the enzymatic activity were registered for glucose-6-phosphate dehydrogenase and hexokinase, accompanied by increases in the activity of glucose-6-phosphatase, as evidenced in the experimental model of streptozotocin-induced diabetes. The aloxanic diabetes induced significant alterations of lipidic profiles in rat compared to the non-diabetic group, as follows: total cholesterol increased by 51.19%, plasma triglycerids increased by 50.30% and LDL-cholesterol increased by 59.46%.

The designed pharmacological model can assist further in determining the anti-diabetic potential of newly synthesized compounds or plant extracts.

Rezumat

A fost realizat un model farmacologic experimental de diabet indus cu aloxan la șobolani albi, masculi și femele din sușa Wistar. S-a utilizat o colectivitate formată din 312 animale, cu greutatea medie inițială de $225,1 \pm 31,48$ g și cu o valoare medie a glicemiei bazale de $103,0 \pm 34,22$ mg/dL. Inducerea diabetului s-a făcut prin administrarea intraperitoneală a unei doze de 130 mg/kg corp aloxan. Rezultatele experimentale au arătat că în colectivitatea testată, un procent de 66,34% animale au devenit diabetice, având o valoare medie a glicemiei de $450,4 \pm 129,7$ mg/dL. Pentru lotul diabetic, s-au înregistrat scăderi ale activității glucozo-6-fosfat dehidrogenazei și hexokinazei, însoțite de creșteri ale activității glucozo-6-fosfatazei, fenomene evidențiate și în modelele experimentale de diabet indus cu streptozotocin. Diabetul aloxanic a indus modificări semnificative ale profilului lipidic al șobolanilor, astfel: creșterea colesterolului total cu 51,19%, creșterea trigliceridelor plasmatice cu 50,30%, creșterea LDL colesterolului cu 59,46% față de lotul non-diabetic.

Modelul farmacologic poate servi ulterior la determinarea potențialului antidiabetic al substanțelor de sinteză noi sau a extractelor din plante.

Keywords: diabetes, alloxan, triglycerides, cholesterol, LDL-cholesterol, glucose-6-phosphate dehydrogenase, glucose-6-phosphatase, hexokinase.

Introduction

Noninsulin-dependent diabetes mellitus characterizes a heterogeneous group of dysfunctions caused by resistance to insulin and abnormal secretion of insulin, having an effect of increase in blood glucose concentration [10]. Up to now several dysfunction subtypes were identified, having as causality genetic defects (mitochondrial diabetes, severe insulin resistance syndrome), with contribution from external factors, responsible for disease progression and complications [13, 19].

The experimental animal models used in the study of type II diabetes are more complex and heterogeneous than those used in the study of type I diabetes, thus resembling the variability of cases in humans. As a result, in some animals insulin resistance predominates while in others it predominates beta cells destruction. Animal models, in which glucose intolerance is an integrant part of a larger phenotype including obesity, dyslipidemias and arterial hypertension, are essential in offering more comprehensive data on noninsulin-dependent diabetes. Selection of individuals with diabetogenic potential and their multiplication in laboratory led to obtaining the strains employed nowadays in such studies. In the following, several animal experimental models are presented [14, 27].

Alloxan and streptozotocin are two of the most used substances for inducing diabetes mellitus in animals, frequently employed in pre-clinical research of diabetes. Both are cytotoxic analogues of glucose and, although they act on different paths, their selective action on pancreatic beta cells is identical [15].

The diabetogenic effect of alloxan is due to its rapid absorption in pancreatic beta cells and the formation of oxygen reactive species [8]. Formation of oxygen reactive species is preceded by alloxan reduction, as it exhibits an increased affinity to substrates containing –SH group – reduced glutathione, cysteine, certain proteins, even enzymes being susceptible to its action [16]. Alloxan's reduction product, dialuric acid, is re-oxidized to alloxan, the redox cycle being responsible for superoxide radicals' release.

Glucokinase (hexokinase IV) is the most sensitive thiolic enzyme from pancreatic beta cells, with a 50% maximal inhibitory concentration MIC50 between 1 and 10 $\mu\text{mol/L}$. At higher concentrations, alloxan can affect more essential enzymes, proteins and cellular functions [12].

The result of glucokinase inhibition by alloxan is decreased by glucose oxidation and tissue plasminogen activator (TPA) generation, thus suppressing de TPA-dependent signaling that stimulates insulin secretion [17].

The present experimental pharmacological model was designed to investigate alloxan effects on glycaemia, lipid profiles and enzymes involved in diabetes mellitus (hexokinase, glucose-6-phosphatase and glucose-6-phosphate dehydrogenase) in white male and female Wistar rats. The designed model can assist further in determining possible anti-diabetic action of newly synthesized compounds [20] or plant extracts.

Materials and Methods

A group of 312 white, males and females Wistar rats were used, having a mean weight of 225.1 ± 31.48 g-bw, and alloxanic diabetes was induced by administering a dose of 130 mg/kg-bw ip.

Animals were weighed initially and at 48 hours following alloxan administering in order to quantify alloxan influence on bodyweight.

From the collectivity two groups were selected (14 animals/group): diabetics and normoglycaemics.

Diabetic animals were treated for 7 days consecutively with distilled water (1 mL/100 g-bw, p.o.). In the seventh day, one and a half hour following distilled water administration, animals were sacrificed and parameters were determined: glycaemia, total cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides. Liver was harvested and enzymatic activities were determined for glucose-6-phosphatase, glucose-6-phosphate dehydrogenase and hexokinase.

In parallel a non-diabetic group was similarly treated, performing the same determinations.

Method for diabetes induction with alloxan

Induction of noninsulin-dependant diabetes was performed by administering a dose of 130 mg/kg-bw alloxan *i.p.* The solution of alloxan 13% was prepared in normal saline. 48 hours after alloxan administration, glycaemia was determined with an ACCU-CHEK Active apparatus (Roche Diagnostics GmbH, D68298 Mannheim, Germany). Blood was drawn from tail veins by puncturing. Determination permitted selecting diabetic animals that had glycaemia over 200 mg/dL.

Method for glycaemia determination

The glucose assay [3, 4] was performed by the enzymatic method with glucose oxidase, employing the principle of glucose oxidation to

gluconic acid with H_2O_2 release, which, in the presence of phenol and of 4-aminoantipyrine, forms a colored complex (4-p-benzoquinono-monoimino-phenazone) suitable for colorimetry.

Method for total cholesterol determination

It consists in oxidizing the cholesterol and forming cholesterol-3-one [22, 23, 24]. The latter, in the presence of aminoantipyrine and of phenol, leads to red-colored quinoimine, the intensity of color being proportional to cholesterol concentration.

Method for HDL-cholesterol determination

Low-density (LDL) or very low-density (VLDL) lipoproteins from the samples, precipitate with phosphowolframic acid in the presence of magnesium ions. The supernatant obtained following centrifugation contains high-density lipoproteins (HDL) in which cholesterol is enzymatically determined [18, 25].

Method for triglycerides determination

The method consists in hydrolyzing triglycerides to glycerol, which forms a quinoiminic complex in the presence of 4-aminoantipyrine and of 4-chlorophenol. Intensity of color is proportional to triglycerides concentration [7, 9, 11].

Method for determination of glucose-6-phosphate dehydrogenase activity

The enzymatic activity is measured through the formation of NADPH by measuring the variation of optical density at 340 nm for 5 minutes [3, 5].

Method for determination of glucose-6-phosphatase activity

Inorganic phosphate formed by enzymatic reaction is determined by reacting with ammonium molybdate; the resulted ammonium phosphomolibdate, treated with a reducing agent solution (1-amino 2-naphtyl 4-sulphonic acid or eikonogen, in $\text{NaHSO}_3 - \text{Na}_2\text{SO}_3$ environment), is transformed in molybdenum blue (combination of Mo_2O_5 with MoO_3), whose intensity of coloration is directly proportional with phosphate concentration and reflects catalytic activity of the enzyme [3, 5].

Method for determination of hexokinase activity

The method consists in monitoring NAD^+ reduction (formation of $\text{NADH} + \text{H}^+$) by determining the variation of optical density at 340 nm for 4 minutes as it measures enzymatic activity [1].

Results and Discussion

Statistical calculus was performed using GraphPad Prism 5.00 for Windows (GraphPad Software - San Diego, California, USA).

Response normality was verified by D'Agostino&Pearson test. Comparison to initial values was performed using Mann Whitney test for abnormal distribution or using *t* Student test for normal distribution.

Experimental results have shown that within the tested collectivity a percentage of 66.34% of animals became diabetic (figure 1, table I), with a mean glycaemia of 450.4 ± 9.017 mg/dL (mean $\pm$ standard error of the mean $M \pm \text{SEM}$). For these animals a 3.40% reduction of bodyweight was also registered against initial weight values ($p=0.0362$). Glycaemic values compared to initial ones for the diabetic collectivity increased by 336.01%.

A percentage of 33.66% of animals became hyperglycemic (Figure 1) following i.p. alloxan administration, glycaemia values increasing by 50.97% compared to basal ones. A 2.76% reduction in bodyweight was registered for this group also but the decrease was not significant compared to the initial ($p=0.1099$).

Table I

Variation of bodyweight and glycaemia for collectivity animals, basal and at 48 hrs following alloxan administration (130 mg/kg-bw, i.p.)

Parameter	M $\pm$ SEM	D'Agostino&Pearson	Mann Whitney
Initial weight (g)/collectivity	225.1 $\pm$ 1.752	Normal distribution	
Weight at 48 hrs after alloxan administration (g)/collectivity	218.0 $\pm$ 1.904	Normal distribution	0.0109
Initial weight (g)/diabetic collectivity	223.4 $\pm$ 2.293	Normal distribution	
Weight at 48 hrs after alloxan administration (g)/diabetic collectivity	215.8 $\pm$ 0.406	Normal distribution	0.0362
Initial glycaemia (g)/collectivity	103.0 $\pm$ 2.480	Normal distribution	
Glycaemia at 48 hrs after alloxan administration (g)/collectivity	343.3 $\pm$ 9.820	Normal distribution	< 0.0001
Initial glycaemia (g)/ diabetic collectivity	103.3 $\pm$ 0.519	Normal distribution	
Glycaemia at 48 hrs after alloxan administration (g)/ diabetic collectivity	450.4 $\pm$ 9.017	Normal distribution	< 0.0001
% diabetic animals	66.34%		
% non-diabetic animals	33.66%		

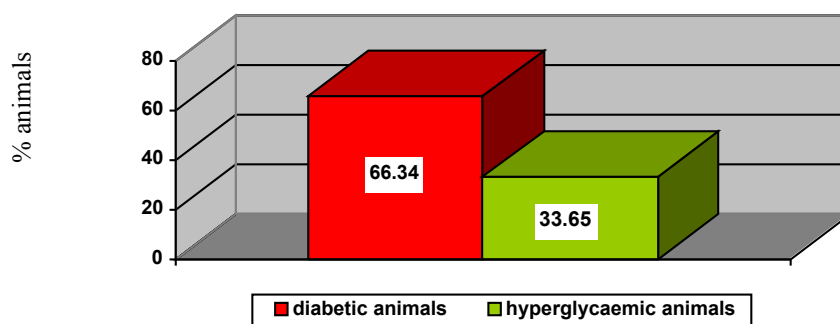


Figure 1

Frequency of response by diabetes or hyperglycemia in collectivity, following alloxan administration, 130 mg/kg-bw, i.p.

After 7 days of distilled water administration, the glycaemia of non-diabetic (control) group has not modified significantly ($p=0.17$). For diabetic animals, glycaemic values decreased by 31.28% against initial values after alloxan administration, animals still remaining diabetic, with actual confirmation in the mean glycaemic value/group: 228.6 ± 38.16 mg/dL ($M \pm SD$).

Table II

Variation of glycaemia compared to initial for non-diabetic and diabetic group

Parameter	Non-diabetic control		Diabetic group	
Glycemia (mg/dL)	Initial	After 7 days administration of distilled water	Glycaemia at 48 hrs after alloxan administration	After 7 days administration of distilled water
$M \pm SD$	112.1 ± 24.58	112.3 ± 7.857	332.7 ± 124.5	228.6 ± 38.16
D'Agostino & Pearson	Abnormal distribution	Normal distribution	Abnormal distribution	Abnormal distribution
Mann Whitney	-	0.0768 ns	-	-
t Student	-	-	-	0.0060
Effect %/initial		0.17		-31.28
Effect %/non-diabetic group			196.78	103.56

Alloxan-induced diabetes caused alterations in the activity of the three enzymes involved (Figure 2) in the homeostasis of glucidic metabolism:

- *Glucose-6-phosphate dehydrogenase* with the role of transforming glucose down the pentose-phosphate pathway;

- *Glucose-6-phosphatase* which catalyzes the hydrolysis of glucose-6-phosphate and generates glucose and inorganic phosphate;

- *Hexokinase* which catalyzes the phosphorylation of glucose.

For the diabetic group, decreases in activity of glucose-6-phosphate dehydrogenase and hexokinase, accompanied by increases in activity of glucose-6-phosphatase were registered, phenomena also highlighted in experimental models of diabetes induced with streptozotocin (Table III).

In this experimental model, a statistically significant decrease in activity of glucose-6-phosphate dehydrogenase was registered ($p=0.0214$) against non-diabetic group. It has also been noted for the diabetic group a significant Pearson correlation between glycemic values and enzyme activity ($p=0.0159$). Experimental studies demonstrated that, in laboratory animals in which diabetes was induced with streptozotocin, a significant reduction in activity of hexokinase was registered [2, 6, 21].

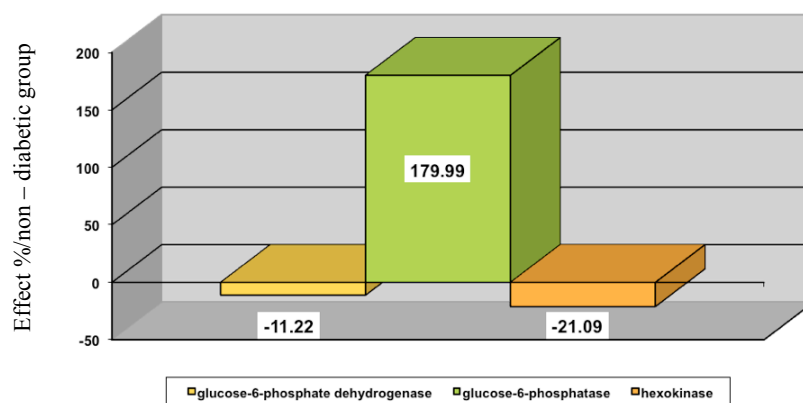
The present study results confirm that enzyme activity is reduced statistically significant ($p=0.03190$) against non-diabetic group (-21.09%), but no significant Pearson correlation was identified between glycemic values and enzyme activity.

Table III

Statistical significance of the alterations of enzymatic activity for diabetic group against non-diabetic group

Group	Glycaemia (mg/dL)	Glucose-6-phosphate dehydrogenase (units/mg protein)	Glucose-6-phosphatase (units/mg protein)	Hexokinase (units/mg protein)
Non-diabetic M $\pm$ SD	112.1 $\pm$ 24.58	1.515 $\pm$ 0.1436	0.3259 $\pm$ 0.3238	0.2821 $\pm$ 0.06536
Diabetic M $\pm$ SD	228.6 $\pm$ 38.16	1.345 $\pm$ 0.2168	0.9125 $\pm$ 0.6223	0.2226 $\pm$ 0.07345
<i>t</i> Student/ non-diabetic	-	0.0214	0.0043	0.0319
Pearson Correlation: glycaemia/ enzymatic activity Non-diabetic	-	0.0502 ns *	0.1743ns	0.2102ns
Pearson correlation: glycaemia/enzymat ic activity Diabetic group		0.0159	0.0002	0.1242
Effect %/ non-diabetic group		-11.22	179.99	-21.09

*ns – non significant

**Figure 2**

Alterations in enzymatic activity for glucose-6-phosphate dehydrogenase, glucose-6-phosphatase and hexokinase for diabetic group compared to non-diabetic group

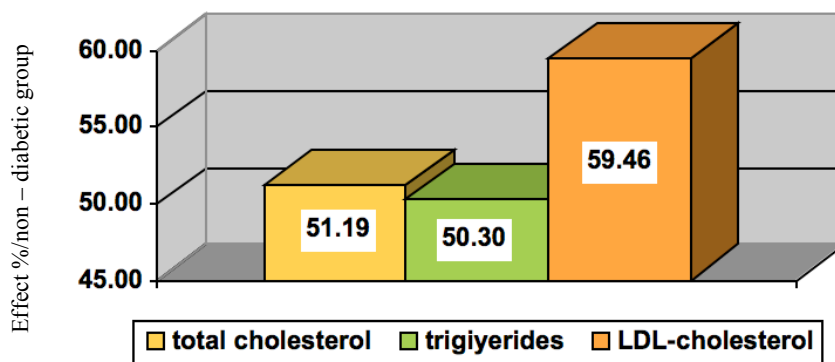
Experimental results (Table IV, Figure 3) demonstrate that alloxan-induced diabetes produce significant alterations in lipid profiles of rats, as follows:

- Increases total cholesterol by 51.19% compared to non-diabetic group ($p=0.0005$)
- Increases plasma triglycerides by 50.30% ($p=0.0002$)
- Increases LDL-cholesterol by 59.46% ($p=0.0160$)
- Insignificantly increases HDL-cholesterol by 5.28% compared to non-diabetic group

Table IV

Statistical significance of lipid profiles alterations for the diabetic group compared to non-diabetic group

Group	Total cholesterol (mg/dL)	Triglycerides (mg/dL)	HDL-c (mg/dL)	LDL-c (mg/dL)
Non-diabetic group M $\pm$ SD	95.36 $\pm$ 27.12	54.52 $\pm$ 17.87	36.32 $\pm$ 10.28	57.18 $\pm$ 35.63
Diabetic group M $\pm$ SD	144.3 $\pm$ 37.71	81.96 $\pm$ 15.86	38.23 $\pm$ 11.51	91.80 $\pm$ 35.45
<i>t</i> Student/ non-diabetic group	0.0005	0.0002	0.6524 ns	0.0160
Effect %/non-diabetic group	51.19	50.30	5.28	59.46

**Figure 3**

Altered lipid profiles for diabetic group, compared to non-diabetic group

Conclusions

An experimental model of alloxan-induced diabetes was created by administering a dose of alloxan 130 mg/kg-bw, i.p. The experiments results have showed that, within tested collectivity, a percentage of 66.34% animals have become diabetics following the administration of the forementioned dose.

The alloxan-induced diabetes has determined modifications in liver enzymes, as follows: decreases in activity for glucose-6-phosphate dehydrogenase and hexokinase and increases in activity for glucose-6-phosphatase.

It also altered significantly lipid profiles in rats, as follows: increased total cholesterol, increased plasma triglycerides and increased LDL-cholesterol.

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