EXPERIMENTAL PHARMACOLOGICAL MODEL OF DIABETES INDUCTION WITH ALOXAN IN RAT

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Abstract

An experimental pharmacological model of diabetes induced with alloxan 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 alloxan. 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 evidentiated in the experimental model of streptozotocin-induced diabetes. The alloxanic 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 alloxan la şobolani albi, masculi şi femele din suşa Wistar. S-a utilizat o colectivitate formată din 312 animale, cu greutatea medie iniţială de 225,1±31,48 g şi cu o valoare medie a glicemiei bazale de 103,0±34,22 mg/dL. Inducerea diabetului s-a făcut prin administrarea intraperitoneală a unei doze de 130 mg/kg corp alloxan. 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±129,7 mg/dL. Pentru lotul diabetic, s-au înregistrat scăderi ale activităţii glucozo-6-fosfat dehidrogenazei şi hexokinazei, însoţite de creşteri ale activităţii glucozo-6-fosfatazei, fenomene evidenţiate şi în modelele experimentale de diabet indus cu streptozotocin. Diabetul aloxanic a indus modificări semnificative ale profilului lipidic al şobolanilor, astfel: creşterea colesterolului total cu 51,19%, creşterea trigliceridelor plasmatice cu 50,30%, creşterea LDL-colesterolului cu 59,46% faţă 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 µmol/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 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 alloxan 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 \( \text{H}_2\text{O}_2 \) 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-chlorphenol. 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-naphthyl 4-sulphonic acid or eikonogen, in \( \text{NaHSO}_3 – \text{Na}_2\text{SO}_3 \) environment), is transformed in molybdenum blue (combination of \( \text{Mo}_2\text{O}_5 \) with \( \text{MoO}_3 \)), 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 NADH + 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 ± standard error of the mean M±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>
<thead>
<tr>
<th>Parameter</th>
<th>M±SEM</th>
<th>D’Agostino&amp;Pearson</th>
<th>Mann Whitney</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial weight (g)/collectivity</td>
<td>225.1±1.752</td>
<td>Normal distribution</td>
<td></td>
</tr>
<tr>
<td>Weight at 48 hrs after alloxan administration (g)/collectivity</td>
<td>218.0±1.904</td>
<td>Normal distribution</td>
<td>0.0109</td>
</tr>
<tr>
<td>Initial weight (g)/diabetic collectivity</td>
<td>223.4±2.293</td>
<td>Normal distribution</td>
<td></td>
</tr>
<tr>
<td>Weight at 48 hrs after alloxan administration (g)/diabetic collectivity</td>
<td>215.8±0.406</td>
<td>Normal distribution</td>
<td>0.0362</td>
</tr>
<tr>
<td>Initial glycaemia (g)/collectivity</td>
<td>103.0±2.480</td>
<td>Normal distribution</td>
<td></td>
</tr>
<tr>
<td>Glycaemia at 48 hrs after alloxan administration (g)/collectivity</td>
<td>343.3±9.820</td>
<td>Normal distribution</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Initial glycaemia (g)/ diabetic collectivity</td>
<td>103.3±0.519</td>
<td>Normal distribution</td>
<td></td>
</tr>
<tr>
<td>Glycaemia at 48 hrs after alloxan administration (g)/ diabetic collectivity</td>
<td>450.4±9.017</td>
<td>Normal distribution</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>% diabetic animals</td>
<td>66.34%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% non-diabetic animals</td>
<td>33.66%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
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± SD).

**Table II**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Non-diabetic control</th>
<th>Diabetic group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycemia (mg/dL)</td>
<td>Initial</td>
<td>Glycaemia at 48 hrs after alloxan administration</td>
</tr>
<tr>
<td>M± SD</td>
<td>112.1±24.58</td>
<td>332.7±124.5</td>
</tr>
<tr>
<td>D’Agostino &amp; Pearson</td>
<td>Abnormal distribution</td>
<td>Abnormal distribution</td>
</tr>
<tr>
<td>Mann Whitney</td>
<td>-</td>
<td>0.0768 ns</td>
</tr>
<tr>
<td>t Student</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Effect %/initial</td>
<td>0.17</td>
<td>-</td>
</tr>
<tr>
<td>Effect %/non-diabetic group</td>
<td>196.78</td>
<td>103.56</td>
</tr>
</tbody>
</table>

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 phosphorilation 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

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

*ns – non significant
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

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

References

10. Ionica, FE; Pisoschi, C; Negres, S; Nikos, RF; Tarata, M; Popescu, F. Atorvastatin influence on glycemic control in patients with type 2 diabetes mellitus. *Farmacia*, 2010, 58 (6), 728-734
19. Manuscript received: December 12th 2012.