Proposal open for discussion: defining agreed diagnostic procedures in experimental diabetes research

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Abstract

**Background:** Animal experimentation has a long tradition in diabetes research and has provided invaluable benefits with regard to insulin discovery and treatment assessment.

**Methods:** The review focuses on chemical-induced diabetes in rats and surveys the protocols of diabetes induction, diabetes diagnosis, and glucose tolerance evaluation in a selection of recent research.

**Results:** This brief review of techniques in experimental diabetes highlights that there is no uniformity, whereas standardisation of procedures is desirable so that comparability will exist among experiments carried out in different settings.

**Conclusions:** On this basis, questions are put and standards are proposed. It would be a platform to promote the exchange of ideas through expert consultation about practical issues related to animal research and a basis on which standards can be set according to user requirements and animal respect.

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**Keywords:** Experimental diabetes; OGTT; IVGTT; IPGGT; Standardisation

1. Introduction

Animal experimentation has a long tradition in diabetes research. In 1890, Von Merhing and Minkowski induced acute diabetes mellitus by removing the pancreas in a dog (Minkowski, 1989). Apart from partial or total pancreatectomy, non-surgical methods of inducing hyperglycaemia exist. The five major classes of diabetogenic agents are chemicals, biological agents, peptides, potentiators, and steroids (Lenzen and Bailey, 1984; Wright and Lacy, 1988; Wilson and LeDoux, 1989; Herold et al., 1996; Lewis et al., 2000; Valerio et al., 2004; Izquierdo-Vega et al., 2006; van der Werf et al., 2007).

Among the animal models available, rodents have been studied extensively for several reasons: economic considerations, short generation time, inherited forms of hyperglycaemia, and/or obesity. Indeed, spontaneous animal models of type 1 diabetes (T1D) and type 2 diabetes (T2D) also exist (Rees and Alcolado, 2005; Chen and Wang, 2005; Yang and Santamaria, 2006). Spontaneously T1D animals, that have been inbred in laboratories by selecting for hyperglycaemia, include the most commonly used non-obese diabetic (NOD) mouse and bio breeding (BB) rat, but also Long Evans Tokushima lean rat, New Zealand white rabbit, Keeshond dog, Chinese hamster, and Celebes black ape. Animal models of T2D are heterogeneous. They include not only animals with single gene mutations, such as ob/ob mouse (leptin deficient), db/db mouse (leptin resistant), Zucker (fa/fa) rat (leptin resistant), and agouti strains, but also many other examples of the insulin resistant syndrome and/or beta-cell failure. Recently, molecular biological techniques have produced genetically engineered mouse models, including knockout and transgenic mice. Gene targeting is the process by which a single gene may be disrupted in an embryonic stem cell and ‘knockout’ animals can be produced. Transgenics incorporates modified genes into the pronucleus of a zygote that are randomly incorporated into the genome and transmitted to some offspring.

Undoubtedly, animal models of diabetes have provided invaluable benefits with regard to insulin discovery and...
treatment assessment. One of the guiding principles of animal research is to use the lowest possible animal: the smaller the animal, the more manageable and cheaper the experiment. However, drawbacks are the lack of reproducible paradigms of human diabetic complications (Gabra et al., 2006; Tesch and Nikolici-Paterson, 2006) and the disappointing results of studies aimed at preventing T1D that were successful in rodents (Leiter and von Herrath, 2004; Roep and Atkinson, 2004). It was argued that rodents might not adequately reflect the human situation (Mestas and Hughes, 2004; Yang and Santamaria, 2006).

Apart from and before any other consideration, standardisation of research methods is a prerequisite when comparing intervention related outcomes. Valid conclusions about differences in pharmaceutical efficacy or survival between animals and over time can be drawn only from comparable datasets of high quality.

The review focuses on chemical-induced diabetes in rats and surveys the protocols of diabetes induction, diabetes diagnosis, and glucose tolerance evaluation in a selection of recent research. On this basis, questions are put and standards are proposed. It would be a platform to promote the exchange of ideas through expert consultation about practical issues related to animal research and a basis on which standards can be set according to user requirements and animal respect.

2. Sine qua non

2.1. European Union legislation on the protection of animals used in scientific experiment

Just in the World Medical Association’s Declaration of Helsinki of 1964 (successively revised and amended) rule 12 provides that “appropriate caution must be exercised in the conduct of research which may affect the environment, and the welfare of animals used for research” (http://www.wma.net/e/ethicsunit/helsinki.htm).


2.2. The four R’s

In 1959, the zoologist William Russell and the microbiologist Rex Burch proposed the three R’s of research: replacement, reduction, and refinement (Russell and Burch, 1959). Replacement technique is any scientific method employing non-sentient material, which may replace methods that use conscious living vertebrates. In relative replacement, animals are still required, though in actual experiment they are exposed, probably or certainly not to distress at all. In absolute replacement, animals are not required at all at any stage. Tissue culture forms a bridge from the relative to the absolute replacement.

Reduction refers to methods that enable researchers to reduce the numbers of animals used in the research, first of all by proper application of statistical information. With regard to the likelihood of statistical significance in medical research, there are important statistical and ethical implications in the choice of sample size for a study (Douglas, 1980). The power of a significance test, that is a measure of the ability to detect a true difference of clinical importance, may be calculated

<table>
<thead>
<tr>
<th>Author</th>
<th>Dose (mg/kg)</th>
<th>Route</th>
<th>BG threshold (mg/dl)</th>
<th>Meter</th>
<th>Days after STZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bae et al. (2006)</td>
<td>45</td>
<td>i.v.</td>
<td>&gt;250</td>
<td>Y</td>
<td>7</td>
</tr>
<tr>
<td>Chang et al. (2006)</td>
<td>70</td>
<td>i.v.</td>
<td>&gt;350</td>
<td></td>
<td>7</td>
</tr>
<tr>
<td>Cho et al. (2006)</td>
<td>40 on 2 days</td>
<td>i.p.</td>
<td>&gt;300</td>
<td></td>
<td>96</td>
</tr>
<tr>
<td>Dimo et al. (2007)</td>
<td>55</td>
<td>i.v.</td>
<td>&gt;350</td>
<td>Y</td>
<td>4</td>
</tr>
<tr>
<td>Huang et al. (2006)</td>
<td>60</td>
<td>i.v.</td>
<td>&gt;270</td>
<td>Y</td>
<td>3</td>
</tr>
<tr>
<td>Iwai et al. (2006)</td>
<td>50</td>
<td>i.p.</td>
<td>–</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Kang et al. (2006)</td>
<td>50</td>
<td>i.p.</td>
<td>–</td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>Kim et al. (2006)</td>
<td>50</td>
<td>i.v.</td>
<td>≥300</td>
<td>Y</td>
<td>2</td>
</tr>
<tr>
<td>Kumar et al. (2006)</td>
<td>60</td>
<td>i.p.</td>
<td>≥240</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Li (2007)</td>
<td>50</td>
<td>i.p.</td>
<td>≥350</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Liu et al. (2006)</td>
<td>65</td>
<td>i.v.</td>
<td>–</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Minamizono et al. (2006)</td>
<td>45</td>
<td>i.v.</td>
<td>–</td>
<td>Y</td>
<td>7–21</td>
</tr>
<tr>
<td>Murugesh et al. (2006)</td>
<td>65</td>
<td>i.p.</td>
<td>≥225</td>
<td>Y</td>
<td>5</td>
</tr>
<tr>
<td>Peredo et al. (2006)</td>
<td>55</td>
<td>i.p.</td>
<td>≥287</td>
<td>Y</td>
<td>7</td>
</tr>
<tr>
<td>Pushparaj et al. (2007)</td>
<td>60</td>
<td>i.p.</td>
<td>&gt;300</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Ugochukwu and Figgers (2007)</td>
<td>35</td>
<td>i.p.</td>
<td>≥200</td>
<td>Y</td>
<td>7</td>
</tr>
<tr>
<td>Yazdanparast et al. (2007)</td>
<td>40</td>
<td>i.p.</td>
<td>≥270</td>
<td>?</td>
<td>7</td>
</tr>
<tr>
<td>Yoshida et al. (2006)</td>
<td>40</td>
<td>i.v.</td>
<td>–</td>
<td></td>
<td>7</td>
</tr>
</tbody>
</table>

Diabetes was confirmed by measuring blood glucose (BG) levels over a variable period (from 1 to 96 days) after STZ injection. BG was measured by using portable meters (Y: yes; ?: assay method not specified). The animals with BG equal or above the indicated threshold were considered to be diabetic.
prospectively. If the smallest difference of clinical relevance can be specified, one can calculate the sample size necessary to have a high probability of obtaining a statistically significant result. Common requirements are (1) the variable is normally distributed and (2) an estimate of the standard deviation is available.

Refinement is the best quality care that can be afforded the animal. A fourth R has been proposed (Bark, 1995) as being responsibility toward research animals. It focuses new facility design and facility renovation performing approved experimentation in a manner as distress free as possible.

A 2005 report presented a consensus statement produced by a working party that represented a wide range of views on animal research. It identified agreement on important issues and made recommendations for improving the quality of the debate and promoting the three R’s (Perry, 2007).

3. Botanicals and herbal preparations

3.1. Standardisation in the manufacture of herbal medicinal products

WHO’s guidelines address specific issues connected with the manufacture of certain types of pharmaceutical products (World Health Organization, 2003, 2005). They provide general and minimum technical requirements for quality assurance and control in manufacturing herbal medicines as a reference to WHO member states. Herbal medicines include herbs (crude materials), herbal materials (herbs, fresh juices, gums, oils, resins, and dry powders of herbs), herbal preparations (comminuted or cut herbal materials, extracts, tinctures, and fatty oils) and finished herbal products. The reference standard may be a botanical sample of the herbal material, a sample of the herbal preparation, a chemically defined substance; it should be stored under appropriate conditions to prevent degradation. Unlike conventional pharmaceutical products, herbal medicines are prepared from herbal materials, which can be obtained from varied geographical or commercial sources. Quality assurance requires the use of modern analytical techniques as well as the control of starting materials, storage, and processing. The specifications for starting herbal materials, for herbal preparations, and finished herbal products are intended to define the quality and focus on those characteristics are found to be useful in ensuring the safety and efficacy.

According to WHO’s guidelines, the specifications for herbal materials/preparations should include the family and botanical name of the plant, details of the source of the plant, which part of the plant is used and its state, a description of the plant material, identifications tests for known active ingredients or markers, limit tests, and suitable methods for the determination of likely contaminants. The processing methods should describe the different operations performed on the plant material (drying, crushing, milling, etc.) and the time and temperatures required in the drying process. For the production of processed extracts, one should specify details of any vehicle or solvent used and the times and temperatures observed during extraction, any concentration stages and methods that have been required. The constituents with known therapeutic activity in herbal material, herbal preparations, and finished herbal products may be identified and/or quantifiable or not. Identification methods should be based on physical, macroscopic and microscopic tests, chromatographic procedures, and chemical reactions. Some stability data should be available.

3.2. Active extract, active compound

Plant preparations include powdered plant material, extract, tincture, and fatty or essential oils. They may be produced by extraction, fractionation, purification, concentration, or other physical or biological procedure. The herbal materials or herbal preparations are considered to be active ingredients of herbal medicines. Therapeutic activity refers to any beneficial alteration or regulation of the physical and mental status of the body, yet safety and efficacy of herbal medicine with a well-documented history of traditional use should be evaluated. If constituents with therapeutic activities are known, the preparation should be standardised to contain a defined amount of the active ingredients. Markers are chemically defined constituents of an herbal material utilised for control purposes, that may or may not contribute to the clinical efficacy. There is a need to approach scientific proof and clinical validation with chemical standardisation, biological assays, animal models, and clinical trials, as described in detail by Ong (2004) and in other review articles there cited. However, standardisation of herbal extract to create a uniform product for clinical trial is a crucial subject under discussion since, according to herbalist’s perspective, quality and effectiveness of traditional medicines could be compromised (Kumar and Bhatnagar, 2006; Tierra, 2007). In the case of active constituent extract, the high degree of concentration may cause a partial representation of all normally occurring constituents thus limiting the broad range of traditionally known properties. Some constituents could be lost that are even more effective than the presumed active compounds. In the case of chemical marker compounds, their use encourages the misconception of herbs as a substitute for drugs. Different standardisation procedures may produce different finished products; furthermore, manufacture sometimes involves the use of toxic solvents. Another important (yet neglected) consideration concerns the amount of herb necessary to obtain the active extract or the single biochemical constituent: herbalists do not foster harvesting of wild herbs, yet wild herbs are considered to be superior to cultivated herbs.

4. Toxin-induced diabetes

4.1. Streptozotocin (1-methyl-1-nitroso-3-[(2S,3R,4R, 5S,6R)-2,4,5-trihydroxy-6-(hydroxymethyl)oxan-3-yl]urea)

STZ is an antibiotic that is produced by Streptomyces achomogenes and induces both type 1 and type 2 diabetes mellitus in adult rats. Solutions should be prepared just before use, since the product is unstable; maximum solution stability is at pH 4. The most frequently used dose of this drug is about 65 mg/kg b.w. (Szkudelski, 2001). Similar doses are effective after intraperitoneal administration. STZ given in multiple low doses in the
mouse induces T1D by the activation of immune mechanisms (Ziegler et al., 1984). T2D may be induced in rats by intravenous or intraperitoneal treatment with 100 mg/kg b.w. on the day of birth (Szkudelski, 2001); since 8–10 weeks of age rats manifest mild hyperglycaemia, impaired glucose tolerance, and a loss of beta-cell sensitivity to glucose. Neonatal treatment with STZ leads to a severe reduction in total beta-cell mass (by 90% on day 3), followed by spontaneous yet limited beta-cell regeneration (from days 4 to 7) (Movassat and Portha, 1999).

Examination of recent selected articles (Table 1) evidences that the dose of STZ ranged from 40 to 70 mg/kg intravenously, and from 35 to 65 intraperitoneally (except Cho et al., 2006, who gave animals 40 mg/kg on two consecutive days). The diabetic state was ascertained by measuring blood glucose concentrations over a wide period of time (2–96 days after STZ treatment). Blood glycaemia was determined by means of a glucometer in over a wide period of time (2–96 days after STZ treatment).

Diabetes was confirmed by measuring blood glucose (BG) levels over a variable period (from 2 to 28 days) after A injection. BG was measured by using portable meters (Y: yes; ?: assay method not specified). The animals with BG equal or above the indicated threshold were considered to be diabetic (–: for not specified datum).

### Table 2

<table>
<thead>
<tr>
<th>Author</th>
<th>Dose (mg/kg)</th>
<th>Route</th>
<th>BG threshold (mg/dl)</th>
<th>Meter</th>
<th>Days after A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ajikumaran Nair et al. (2006)</td>
<td>60</td>
<td>i.v.</td>
<td>400–450</td>
<td>Y</td>
<td>5</td>
</tr>
<tr>
<td>Akamine et al. (2006)</td>
<td>40</td>
<td>i.v.</td>
<td>–</td>
<td>Y</td>
<td>–</td>
</tr>
<tr>
<td>Almeida et al. (2006)</td>
<td>150</td>
<td>i.p.</td>
<td>&gt;350</td>
<td>Y</td>
<td>3</td>
</tr>
<tr>
<td>Bae et al. (2006)</td>
<td>40 on 2 days</td>
<td>i.v.</td>
<td>&gt;250</td>
<td>Y</td>
<td>4</td>
</tr>
<tr>
<td>Carvalho et al. (2006)</td>
<td>40</td>
<td>i.v.</td>
<td>&gt;200</td>
<td>Y</td>
<td>–</td>
</tr>
<tr>
<td>Garcia et al. (2006)</td>
<td>150</td>
<td>s.c.</td>
<td>&gt;200</td>
<td>Y</td>
<td>–</td>
</tr>
<tr>
<td>Kannur et al. (2006)</td>
<td>150</td>
<td>i.p.</td>
<td>Test for glycosuria</td>
<td>Y</td>
<td>–</td>
</tr>
<tr>
<td>Leite et al. (2007)</td>
<td>150</td>
<td>i.p.</td>
<td>&gt;200</td>
<td>Y</td>
<td>3</td>
</tr>
<tr>
<td>Odeta et al. (2006)</td>
<td>120</td>
<td>i.p.</td>
<td>&gt;270</td>
<td>–</td>
<td>2</td>
</tr>
<tr>
<td>Perez et al. (2006)</td>
<td>150</td>
<td>i.p.</td>
<td>220–260</td>
<td>Y</td>
<td>–</td>
</tr>
<tr>
<td>Preet et al. (2006)</td>
<td>150</td>
<td>s.c.</td>
<td>–</td>
<td>–</td>
<td>2</td>
</tr>
<tr>
<td>Raut and Gaikwad (2006)</td>
<td>120</td>
<td>i.p.</td>
<td>&gt;200</td>
<td>Y</td>
<td>15</td>
</tr>
<tr>
<td>Schoenfelder et al. (2006)</td>
<td>200</td>
<td>i.p.</td>
<td>&gt;300</td>
<td>?</td>
<td>3</td>
</tr>
<tr>
<td>Somani et al. (2006)</td>
<td>120</td>
<td>s.c.</td>
<td>&gt;250</td>
<td>Y</td>
<td>7</td>
</tr>
<tr>
<td>Suresh and Das (2006)</td>
<td>75 on 5 days</td>
<td>i.p.</td>
<td>&gt;275</td>
<td>Y</td>
<td>14–21</td>
</tr>
<tr>
<td>Tang et al. (2006)</td>
<td>55</td>
<td>i.v.</td>
<td>&gt;200</td>
<td>–</td>
<td>3</td>
</tr>
<tr>
<td>Zanatta et al. (2007)</td>
<td>60</td>
<td>i.v.</td>
<td>–</td>
<td>Y</td>
<td>3</td>
</tr>
<tr>
<td>Zhang et al. (2006)</td>
<td>150</td>
<td>i.p.</td>
<td>&gt;200</td>
<td>–</td>
<td>3</td>
</tr>
</tbody>
</table>

STZ is taken up by pancreatic beta cells via the glucose transporter GLUT2. Its toxicity depends on the potent alkyllating properties combined with the synergistic action of nitric oxide and reactive oxygen species that contribute to DNA fragmentation. Two hours after injection (80 mg/kg i.p.), hyperglycaemia is observed and blood insulin levels decrease; 6 h later, blood insulin levels increase and hypoglycaemia occurs; finally, stable hyperglycaemia develops (West et al., 1996).

In recent literature (Table 2) the dose of alloxan injected was from 40 to 60 mg/kg intravenously (except Bae et al., 2006, who gave 40 mg/kg on two consecutive days), 120 to 200 intraperitoneally (except Suresh and Das, 2006, who used 75 mg/kg on five consecutive days), and 120 to 150 subcutaneously. Diabetes was confirmed in 14 of 18 articles by determining blood glycaemia: the cutoff was from 200 to 400 mg/dl and a glucometer was used for the measurement in six cases.

4.2. **Alloxan (1,3-diazinane-2,4,5,6-tetraone)**

The diabetogenic agent, alloxan, is a hydrophylic and chemically unstable compound, whose half-life at pH 7.4 and 37°C in phosphate buffer is 1.5 min (Lenzen and Munday, 1991). Alloxan diabetes has been commonly considered as an animal model of type 1 diabetes mellitus (Szkudelski, 2001). Alloxan induces a specific necrosis of pancreatic islets when it is administered parenterally: intravenously, intraperitoneally, or subcutaneously. The most frequently used intravenous dose is 40–60 mg/kg b.w.; the effective dose is two to three times higher when alloxan is given intraperitoneally or subcutaneously. Fasted animals are more susceptible to alloxan.

Toxic activity takes place through alloxan rapid uptake by the beta cells (via the glucose transporter GLUT2), alloxan reduction to dialuric acid together with oxidation of SH groups, inhibition of glucokinase, generation of reactive oxygen species, and disturbances in intracellular calcium homeostasis.

Alloxan action is not selective; the diabetogenic dose of alloxan also decreases rat liver SH groups, impairs peripheral insulin sensitivity, and intensifies lipolysis. Thus, Szkudelski (2001) suggests that a proper period of time should elapse before animal examination to minimise side effects of alloxan toxicity, yet what is a “proper period of time” has not been settled. Two minutes after alloxan administration (75 mg/kg i.v.), a sudden rise in blood insulin concentrations is observed in vivo (Szkudelski et al., 1998) followed by complete suppression of the insulin secretion and hyperglycaemia.
Table 3
Oral glucose tolerance test as was done in rats

<table>
<thead>
<tr>
<th>Author</th>
<th>Dose (g/kg)</th>
<th>Times (min)</th>
<th>Meter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ajikumaran Nair et al. (2006)</td>
<td>3.0</td>
<td>0, 30, 90, 150</td>
<td></td>
</tr>
<tr>
<td>Almeida et al. (2006)</td>
<td>1.5</td>
<td>0, 30, 90</td>
<td></td>
</tr>
<tr>
<td>Iwai et al. (2006)</td>
<td>2.0</td>
<td>0, 30, 60, 120</td>
<td></td>
</tr>
<tr>
<td>Kannur et al. (2006)</td>
<td>3.0</td>
<td>0, 30, 90, 150</td>
<td>Y</td>
</tr>
<tr>
<td>Katsuno et al. (2007)</td>
<td>2.0</td>
<td>0, 15, 30, 60, 120</td>
<td></td>
</tr>
<tr>
<td>Kim et al. (2006)</td>
<td>2.0</td>
<td>−30, 0, 30, 60, 90, 120, 150, 180</td>
<td>Y</td>
</tr>
<tr>
<td>Kuman et al. (2006)</td>
<td>10.0</td>
<td>0, 30, 60, 120</td>
<td></td>
</tr>
<tr>
<td>Li et al. (2007)</td>
<td>2.0</td>
<td>0, 30, 60, 120</td>
<td>Y</td>
</tr>
<tr>
<td>Liu et al. (2006)</td>
<td>1.0</td>
<td>0, 30, 60, 90, 120, 180</td>
<td></td>
</tr>
<tr>
<td>Pushparaj et al. (2007)</td>
<td>3.0</td>
<td>−30, 0, 60, 120, 180</td>
<td></td>
</tr>
<tr>
<td>Rubino et al. (2006)</td>
<td>3.0</td>
<td>0, 10, 30, 60, 120, 180</td>
<td>Y</td>
</tr>
<tr>
<td>Satoh et al. (2005)</td>
<td>5.0</td>
<td>0, 15, 30, 45, 60, 90, 120, 180, 300</td>
<td></td>
</tr>
<tr>
<td>Schoenfelder et al. (2006)</td>
<td>4.0</td>
<td>0, 30, 60, 180</td>
<td></td>
</tr>
<tr>
<td>Somani et al. (2006)</td>
<td>1.5</td>
<td>0, 30, 60, 120</td>
<td>Y</td>
</tr>
<tr>
<td>Sriplang et al. (2007)</td>
<td>3.0</td>
<td>0, 30, 90, 120, 210</td>
<td></td>
</tr>
<tr>
<td>Taniguchi et al. (2006)</td>
<td>2.0</td>
<td>0, 30, 60, 120</td>
<td></td>
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<tr>
<td>Yadav et al. (2006)</td>
<td>2.0</td>
<td>0, 15, 30, 60, 90, 120</td>
<td>Y</td>
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<tr>
<td>Yamazaki et al. (2007)</td>
<td>2.0</td>
<td>−30, 0, 30, 60, 120</td>
<td></td>
</tr>
</tbody>
</table>

The animals received glucose at the indicated doses. Blood glucose levels were measured at the indicated times (min) after glucose loading by using portable meters (Y: yes; ?: assay method not specified).

5. Remarks

5.1. Animal respect

Prolonged blood sugar extremes can cause rats to lose consciousness. Both very high blood sugar (hyperglycemia) and very low blood sugar (hypoglycemia) can lead to a diabetic coma that may be heralded by confusion and convulsions. Left untreated, a diabetic coma is a life-threatening condition. Without therapy, diabetic rats may develop severely elevated blood sugar levels and present increased urine glucose, excessive loss of fluid and electrolytes in the urine, inability to store fat and protein along with breakdown of existing fat and protein stores. This dysregulation, results in the process of ketosis and the release of ketones into the blood. Is seems to be ethically incorrect leaving untreated animals who have blood glycaemia above 450 mg/dl. Exceptionally, some authors specified that those animals with fed plasma glucose higher than 450 mg/dl were excluded from the experiments (Hamamoto et al., 2001).

The same comment is suited also for the initial phase of alloxan-induced insulin release and consequent hypoglycaemia that seems to be never treated. Exceptionally, some authors specified that animals were treated with glucose solution during the first 6 h after the injection to prevent convulsions and death.

Table 4
Intravenous (IVGTT) and intraperitoneal (IPGTT) glucose tolerance tests as were done in rats

<table>
<thead>
<tr>
<th>Author</th>
<th>Dose (g/kg)</th>
<th>Times (min)</th>
<th>Meter</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVGTT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caluwaerts et al. (2006)</td>
<td>1.0</td>
<td>0, 5, 10, 15, 30, 60, 90</td>
<td>Y</td>
</tr>
<tr>
<td>Chang et al. (2006)</td>
<td>0.001</td>
<td>0, 15, 30, 60, 90</td>
<td>Y</td>
</tr>
<tr>
<td>Cheng et al. (2005)</td>
<td>0.5</td>
<td>0, 1, 5, 10, 15, 30, 60, 90</td>
<td>Y</td>
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<tr>
<td>Fernandez-Twinn et al. (2005)</td>
<td>1.0</td>
<td>0, 1, 2, 4, 6, 8, 10, 13, 18, 24, 30, 45, 60, 90</td>
<td>Y</td>
</tr>
<tr>
<td>Ferrari et al. (2005)</td>
<td>0.75</td>
<td>−15, 0, 3, 6, 9, 12, 15, 20, 30, 40, 50, 60, 90</td>
<td>Y</td>
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<tr>
<td>Holness et al. (2005)</td>
<td>0.5</td>
<td>0, 2, 5, 10, 15, 30</td>
<td>Y</td>
</tr>
<tr>
<td>Maiti et al. (2005)</td>
<td>0.5</td>
<td>0, 30, 60, 90, 120</td>
<td>Y</td>
</tr>
<tr>
<td>van de Wall et al. (2006)</td>
<td>0.1</td>
<td>−11, −1, 1, 3, 5, 10, 15, 20, 25, 30, 40, 50</td>
<td>Y</td>
</tr>
<tr>
<td>IPGTT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chanseuma et al. (2006)</td>
<td>1.0</td>
<td>0, 15, 30, 60, 120</td>
<td>Y</td>
</tr>
<tr>
<td>Chung et al. (2005)</td>
<td>2.0</td>
<td>0, 30, 60, 120</td>
<td>Y</td>
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<tr>
<td>Jung et al. (2006)</td>
<td>2.0</td>
<td>0, 15, 30, 45, 60, 90, 120</td>
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<tr>
<td>Liu et al. (2005)</td>
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<td>0, 30, 60, 90, 120</td>
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<tr>
<td>Magaton et al. (2007)</td>
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<td>0, 5, 15, 30, 60, 90</td>
<td>Y</td>
</tr>
<tr>
<td>Metz et al. (2005)</td>
<td>2.0</td>
<td>0, 20, 40, 60, 90, 120</td>
<td>Y</td>
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<tr>
<td>Sondergaard et al. (2006)</td>
<td>2.0</td>
<td>0, 30, 60, 90, 120</td>
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<tr>
<td>Walker et al. (2006)</td>
<td>2.0</td>
<td>0, 30, 60</td>
<td>Y</td>
</tr>
</tbody>
</table>

The animals received glucose at the indicated doses. Blood glucose levels were measured at the indicated times (min) after glucose loading by using portable meters (Y: yes; ?: assay method not specified).
which are common in the hypoglycaemic stage (de Carvalho et al., 2006).

5.2. Analytical considerations

Diabetes induction following toxin administration should be confirmed by blood glucose measurement. If blood is drawn, it should be separated from cells within 60 min or, alternatively, a tube containing a glycolytic inhibitor should be used. Glucose should be measured in plasma by using standardised enzymatic methods (Sacks et al., 2002). Glucose concentrations in plasma are 11% higher than whole blood; glucose concentrations in heparinised plasma can be 5% lower than in serum. The glucose concentrations during oral glucose tolerance test in capillary blood are higher than in venous blood (about 20–25%). A coefficient of variation ≤2.2% has been suggested as a target for imprecision for plasma glucose. Portable meters for measurement of whole blood glucose concentrations are not recommended in the diagnosis of human diabetes because of their imprecision and the variability of results among different glucose meters. No glucose meter has achieved the American Diabetes Association goal of analytical error of <5%.

The diagnosis of diabetes in humans is established according to the revised current criteria (Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 2003):

- symptoms of diabetes and casual plasma glucose ≥200 mg/dl;
- fasting plasma glucose (FPG) ≥126 mg/dl;
- 2 h postload glucose ≥200 mg/dl.

Confirmation by repeat testing on a subsequent day is necessary. However, these criteria are applicable only if methods for glucose analysis exhibit low imprecision at the diagnostic decision limits of 126 and 200 mg/dl, that is not the case of meters.

Open to debate: glucose meters need a drop of whole blood for glucose measurement (less trauma for the animal) yet their poor precision and reproducibility compromise statistical power (need for a larger sample size). What is more convenient from the four R’s viewpoint?

5.3. Glucose tolerance tests

The oral glucose tolerance test (OGTT) evaluates the ability to respond appropriately to a glucose challenge. In adult humans, the 3 h 100 g OGTT is recommended for establishing the diagnosis of gestational diabetes mellitus, whereas the 2 h 75 g OGTT may be used (not routinely) for the diagnosis of diabetes. For children, the dose is 1.75 g glucose/kg b.w. up to 75 g of glucose.

It is a sensitive test to detect disturbances of glucose metabolism, especially when casual and FPG test give ambiguous results. In order to obtain accurate results, precise procedures are required:

- no reduction in carbohydrate intake for at least 3 days;
- the test should be done after an overnight fast (10–14 h);
- 25–30% glucose solution should be administered orally;
- blood glucose should be measured before and at certain time intervals after glucose loading (fasting and 2 h blood samples are indispensable, samples at 30 and 60 min may enhance diagnosis accurateness);
- no food or drink (except water) is allowed during the test.

The dose of anhydrous glucose (25–30% water solution) given to rats was from 1 to 10 g/kg and a glucometer was used for the measurement in 6 of 18 articles. Blood samples (whose number varied from three to nine) were variously collected from baseline (or −30 min) to 90–300 min after the load (Table 3).

Proposal: Using a similar glucose load in animals as in children and establishing that a 2 h plasma glucose cutoff of ≥200 mg/dl should be used. Baseline and 2 h blood samples are sufficient unless research objectives and design justify multiple samples.

When casual and FPG test give unambiguous results and the rat is frankly diabetic, huge glucose loads (as sometimes used) induce a dangerous (for the animal) rise of blood sugar. Thus, they should be avoided in rats that have blood glycaemia above 200 mg/dl.

Question: Which is the maximum load corresponding to humans’ 75 g? In proportion to the weight of an adult male rat (450–500 g), the maximal load could be 0.50 g.

The intravenous glucose tolerance test (IVGTT) has been used as a measure of insulin secretion, and a diminished first phase insulin response (FPIR) is considered a risk factor for human T1D. It was recommended by the Islet Cell Antibody Register Users Study (Bingley et al., 1992). The diabetes prevention trial-type 1 diabetes showed that abnormalities in the 2 h glucose on OGTT are as sensitive and have better specificity for predicting diabetes in the 6-month before diagnosis of diabetes compared with FPIR below the 10th percentile for age. The highest sensitivity is achieved using both tests (Barker et al., 2007). Dextrose was administered at a dose of 0.5 g/kg (maximum 35 g) intravenously over a 3 min period. Blood samples were obtained at −4, −10, 1, 3, 5, 7, and 10 min after the load and analyzed for glucose and insulin. FPIR was the sum of the −1 and 3 min insulin levels.

In rats, the dose of anhydrous glucose was from 0.001 to 1.0 g/kg and a glucometer was used for the measurement in three of eight articles. Blood samples (whose number varied from 5 to 14) were variously collected from baseline (or −15 min) to 30–120 min after the load (Table 4).

Provided that glucose tolerance tests in animal research have rarely the aim of predicting diabetes and, moreover, that the percentile distribution of FPIR in healthy rats is unknown, the IVGTT provides no additional advantage than the oral test in most of experiments.

Proposal: Using a similar glucose load in animals as in humans (in case the experimental design effectively requires IVGTT), and establishing that plasma samples collected from baseline to 10 min should be used.

Question: Which is the maximum load corresponding to humans’ 35 g? In proportion to the weight of an adult male rat, the maximal load could be about 0.25 g.
The intraperitoneal glucose tolerance test (IPGTT) is not used in humans. In rats, glucose dose was from 0.2 to 2.0 g/kg and a glucometer was used for the measurement in three of eight articles. Blood samples (whose number varied from three to seven) were variously collected from baseline to 60–120 min after the load (Table 4).

**Question:** What are the reasons for using IPGTT?

**Proposal:** In case IPGTT is necessary, using a similar glucose load in all animals (which one?), and establishing how many plasma samples should be collected and when.

### 5.4. Study protocol

A protocol is the researcher’s plan of a scientific experiment or treatment. It includes a description of the research design or methodology, the eligibility requirements for study animals and controls, the treatment regimen/s, and the statistical methods of data analysis. Research and evaluation of herbal medicines should follow WHO’s research guidelines for evaluating the safety and efficacy of herbal medicines (World Health Organization, 1993). The primary objectives of non-clinical studies are: (1) to determine whether they support the clinical use of a herbal medicine; (2) to characterise the range of pharmacological actions; (3) to define the chemical characteristics of pharmacologically active natural products and their mechanisms of actions.

Pharmacodynamic and general pharmacological investigations use animal models or bioassays that closely relate to human disease. Test assays can use whole animals, isolated organ or tissues, blood and its components, ex vivo and tissue culture cells, and subcellular constituents. Selection of doses for animal studies should be established by means of a dose–response relationship and in accordance with traditional clinical doses. All studies should include a negative control group (vehicle only) and a positive control group (known drug). Blood/tissue chemistry should include those determinations that may be useful to elucidate the biological activity of an herbal medicine. For example, plasma insulin concentration should be given in addition to glycaemia; liver glycogen and triacyl glycerol levels may help to understand glucose absorption and utilisation, and so on.

Toxicological methods include systemic, local, and special toxicity tests. Acute toxicity test needs a sufficient number of dose levels to determine the lethal dose and an observation interval of at least 7–14 days. In long-term toxicity tests the administration period may range from 2 weeks to 12 months as a function of the expected period of clinical use. In the case of rodents, each group should consist of the same number (at least from 5 to 10) animals per sex.

In most animal researches, the investigators evaluate the effect of an intervention (or condition) and they want to test statistically the null hypothesis, i.e. the proposition that the experimental intervention has no effect. Based on the research design, the study includes appropriate control groups such as animals who do not have the condition or animals who do not get the treatment under study. In this last case, the experimental group receives the treatment to be evaluated, whereas the control group receives a placebo, no treatment or the standard of care. Guidelines underlie the importance of designing animal experiments well, analysing the data correctly, and using the minimum number of animals necessary to achieve the scientific objectives (Festing and Altman, 2002). Randomisation and blinding are rarely reported even though it has been shown that animal experiments carried out without either are five times more likely to report a positive treatment effect (Perel et al., 2007). Random allocation is a carefully planned method of assigning animals into an experimental group and a control group so that the animals in both groups are similar as far as possible with respect to all factors that might affect the outcome. A study is blinded if an investigator blinded to treatment group makes any assessment of the outcome.

Because the safety and effectiveness of drugs are usually tested in animal models before clinical trials are carried out, efforts to avoid bias and random error are important. Moreover, animal models should represent the clinical context as close as possible. For example, animals have to receive doses (g/kg b.w.) of the chemical compound under investigation that could realistically be given a 70 kg man without destroying a forest. Similarly, the dose of the control drug (usually, sulfonylureas in experimental diabetes) should not exceed the highest recommended dose in humans.

### 6. Conclusions

This brief review of techniques in experimental diabetes research highlights that there is no uniformity, whereas standardisation of procedures is desirable so that comparability will exist among experiments carried out in different settings. It should be important to move away from a system that appears to base the choice of experimental protocols, in large part, on individual experience and inclination. It is hoped that current test conditions, procedures, and criteria of interpretation are, although arbitrary, standardised. International experts should provide insightful and valuable comments and suggestions so as to develop an appropriately agreed conclusive document. Indeed, whichever way alternative medical researchers choose to relate their work to evidence-based medicine (Borgerson, 2005), reevaluation of standards of evidence will benefit everyone in the medical community.

### References


