Going Down the Rabbit Hole – Why are Answers to Research Questions often Elusive?

Experimental Animals in Research - Challenges, Choices and Conundrums - a Brief Review

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ABSTRACT
Accurate and ethical research findings are expected to add value to the body of knowledge of any disease process. Results produced from ethical research may have relevance to the early detection of a disease, or offer insights in understanding the full spectrum of a particular disease.

For decades, various animal models have been used to investigate disease processes, yet answers to our research questions remain elusive. Metabolic syndrome and diabetes mellitus have similar aetiologies, often sharing clinical manifestations such as insulin resistance and obesity. Both these entities are considered a health crisis today, due to their prevalence, as well as their associated co-morbidities, and as such, are often focuses of research.

In this brief review, these two disease processes were used as examples to highlight the dissimilar structures of both human and animal physiology, and how this may influence the choice of the animal models used. Irrespective of the disease processes being researched, a detailed literature search is necessary in order to understand these animal model choices. These choices can for example affect and impact the research results, as well as cloud the interpretation these results.

KEYWORDS: animal models, diabetes, insulin resistance, metabolic syndrome, obesity

INTRODUCTION
Animal models are often used to investigate disease processes when attempting to develop prevention and better treatment options for humans. Understanding the disease process itself may pose a challenge in the selection of an optimal animal model. For these reasons, either the characteristics of the model should mirror the pathophysiology or natural history of the condition, or the model should mirror aetiologies to, and develop similar complications of the human condition. As scientists, when designing a research protocol or extrapolating information from literature, it is easy to focus on a single disease parameter of interest. Examples are insulin resistance (IR) or obesity, and in the interpretation of information, often within a discipline specific context. One therefore needs to select an animal model that is appropriate, without adversely affecting the overall process or disease condition.

Metabolic syndrome, diabetes, and obesity are multifactorial disease processes dependent on a complex interaction of host genetics, diet, and numerous environmental factors. Metabolic syndrome is defined loosely by parameters of raised blood pressure, raised blood glucose, and high serum triglycerides. Globally, there are five definitions that describe metabolic syndrome, namely, the World Health Organisation (WHO), the Adult Treatment Panel III (ATPIII) Report, European Group for the Study of Insulin Resistance (EGIR), the International Diabetes Federation (IDF) consensus definition and National Cholesterol Education Program (NCEP) definitions. Many proponents advocate that metabolic syndrome and diabetes mellitus (DM) (of which there are six categories) are a continuum of the same pathological process.
The oversimplification of these categories, viz, latent autoimmune diabetes of adulthood (LADA - also called Type 1.5 DM), maturity onset diabetes of the young (MODY), neonatal diabetes (NDM) and gestational diabetes into the traditional subdivisions of DM into Type 1 diabetes mellitus (T1DM) and Type 2 diabetes4 mellitus (T2DM) poorly describes these categories. These two broad conditions (T1DM and T2DM) probably represent the extremes on a range of disorders characterised by chronic hyperglycaemia. Others consider metabolic syndrome and diabetes to be separate entities.5,6 Typically, in diabetes, the same indicators as in those for metabolic syndrome are present, with central (gynoid) obesity an additional risk factor.6 WHO advocates IR as an absolute requirement with two additional risk factors such as body mass index (BMI) and obesity when diagnosing metabolic syndrome.7

The major concerns associated with metabolic syndrome and diabetes relate to their micro- and macro-vascular complications, which contribute greatly to their morbidity.8,9 Progression from pre-diabetes to an overt diabetic state promotes an adverse vascular milieu with accompanying complications occurring over many years.8,9 Some studies report a five-fold risk of developing T2DM and cardiovascular disease (CVD) in individuals diagnosed with metabolic syndrome.8,9 For this reason, the WHO continues to articulate the prevention of CVD as a primary health care target.7,10 Individuals in many low- and middle-income countries are developing risk factors for metabolic syndrome11 where malnutrition and obesity, and by implication, diabetes coexist. Some diabetic patients remain stubbornly non-obese and the absence of weight gain does not explain the proclivity of these patients developing diabetic syndromes. Globally, 45% of all new-onset T2DM cases are diagnosed in paediatric patients,12 with dietary and exercise programs delivering low success rates in attenuating the condition. These findings imply that the conditions of metabolic syndrome and diabetes are not simple issues of energy and caloric balance. This has left clinicians ill-equipped to treat an “adult” disease15 and its co-morbidities in children. For these reasons, both metabolic syndrome and diabetes form part of the WHO millennial development goals to reduce disease burden.10

In practice, distinguishing between T1DM and T2DM is difficult, especially in children, with a substantial degree of overlap in the syndromes’ clinical presentation.5,14 This overlap includes weight loss in about 20% of adolescents, as well as diabetic ketoacidosis, which is a hallmark of T1DM.15 Interestingly, in adults, high-density lipoproteins (HDL’s) are reduced in T2DM but are not reduced in T1DM. Low-density lipoprotein (LDL) and total cholesterol parameters are relatively normal in most individuals with T2DM.15

Recent evidence of lower grey matter volumes and Alzheimer’s disease in individuals with T2DM, and decreased cognitive impairment in subjects who have metabolic syndrome, further exacerbate the complications of these entities.16 As a result, these entities (metabolic syndrome and diabetes) are often the focus of research, within and across multi-disciplinary groups. The absence of a consensus definition for metabolic syndrome16,17 makes it difficult to accurately diagnose the prevalence of this syndrome.4,17 Additionally, the likelihood in progressively developing diabetes renders challenges to the researcher trying to replicate this human clinical condition in an animal model. The clinical presentation of metabolic syndrome and diabetes are therefore extremely complex with overlapping aetiologies.1,3 In this brief review, these entities are used as examples to highlight the need for careful selection of any animal model because of the anatomical and physiological differences between humans and animals.

Rodents are an attractive research tool and are commonly used.18,19 This is due to their similarity both genetically and physiologically to humans. In some instances, such as with mice, the genome is well described and characterised. Also, fast reproduction rates make small rodent models cost effective, with the added advantage of conducting studies over relatively short periods of time. Environmental models such as streptozotocin (STZ) treatment, partial pancreatectomy (rarely used), as well as exposure to different diets are available to investigate metabolic syndrome and diabetes. Similarly, various transgenic and polygenic mouse models are well developed for investigation of these entities.18

**Chemical induction animal models**

Alloxan and STZ are the two most prominent and widely applied diabetogenic glucose analogues used in animal diabetes research.20,21 These chemical compounds are similar in structure, and also differ slightly in elemental composition. Alloxan is an unstable synthesised pyrimidine derivative. On uptake, it generates a cyclic redox reaction. Streptozotocin is an antimicrobial agent widely used as a chemotherapeutic agent to treat human cancers. The cytotoxic effects of alloxan and STZ are achieved via different pathways.22,23 However, the mechanism of pancreatic beta cells (β-cells) selective response to these chemicals appears to be identical.20 It is well described that IR associated with diabetes causes bi-directional miscommunications between pancreatic β-cells and target tissues such as liver and muscle.15 Both alloxan and STZ accumulate in the β-cells via glucose transporter-2, a specialised protein that allows glucose to move across cell membranes. Other organs that express this transporter, such as the kidney and liver, also incur damage.20,22 It is hypothesised20 that the low levels of glucose transporter-2 in certain tissues results in the preferential uptake of alloxan and STZ. Interestingly, due to the different glucose uptake mechanisms in rats and humans, both alloxan and STZ are non-toxic to the human β-cell.20 This is supported by the absence of diabetes developing as a ‘side effect’ when STZ is used as a chemotherapeutic agent in treating human cancers. As STZ targets rapidly reproducing cancer cells, it also targets other cells that produce rapidly, viz. endothelial cells. Endothelial dysfunction is a well described feature of CVD, both of which are risk factors as a result of diabetes.8

The use of STZ, especially at low doses (45mg/kg) to achieve ablation of pancreatic β-cells, in some aspects, resembles insulin dependent T1DM.20 T1DM differs considerably with the aetiology and mechanism of β-cell destruction as seen in T2DM.12 T2DM mellitus is associated with diminishing pancreatic function and abnormal fuel sensing over time as part of the overall metabolic dysfunction. In typical T1DM in humans, β-cell death results from apoptotic cell death in the absence of insulin leakage and necrotic cell death.24 Necrotic cell death however, is established as one of the mechanisms of action of STZ.20

Extensive STZ-titration studies indicate that not only does sensitivity to the drug differ between strains of animals,25
but that low doses of STZ are unstable as spontaneous recovery of β-cells is known to occur. Furthermore, STZ-induced loss of pancreatic β-cells is characterised by a significant decrease of nerve growth factor (NGF). Small rodent models of STZ-induced diabetic nephropathy show limited resemblance to human diabetic nephropathy with this process unlike the natural history of either T1DM or T2DM human diabetic nephropathy. Butyl alloxan acts in a similar manner to alloxan and STZ, but has a dominant feature of nephrotoxicity of early-onset renal failure. This has been attributed to the differentiating effects of STZ which acts as both an alkylating agent and as an intracellular nitric oxide donor, resulting in hyperglycaemia impacting on renal function and cardiovascular risk profiles. Because of this, controversy on mode of action of these modalities persists and has therefore raised the question as to these chemicals’ validity in mimicking the human conditions of metabolic syndrome and T2DM in rodent models.

Numerous rodent models attempt to establish a causal relationship between metabolic syndrome, diabetes and cognitive function. Models using STZ have usually been in tandem with a therapeutic vehicle to support positive correlations between diabetes and cognitive function. As STZ-induced loss of pancreatic β-cells results in significantly decreased levels NGF, upregulated NGF levels occur in the hippocampus and pituitary, and to a lesser degree, in the hypothalamus and cortex. Nerve growth factor responsive neurons are involved in age-related disorders such as Alzheimer’s disease. The withdrawal of NGF results in neuronal death. Therefore the question remains unanswered if it is the STZ-induced DM or the STZ alone, or both, that are responsible for the negative effects on learning and memory abilities. Streptozotocin has also been linked to inappropriately high levels of N-Methyl-D-Aspartate receptor stimulation and neuronal necrosis in rats. This poses challenges in discerning histological and morphological differences being investigated in the central nervous system, and as such is a poor model within that context. Furthermore, studies using STZ-induction of DM report early tactile allodynia where animals feel pain from stimuli that would otherwise not normally cause pain. This phenomenon can persist unchanged for as long as 24 weeks in humans, typical diabetic neuropathy and allodynia are mechanistically different. Inducing unnecessary pain to animals may create ethical tension, especially in light of inducing a process which is dissimilar to that of the human condition. Other studies report early bilateral cataract development in STZ-treated rats. Similarly, Alloxan causes ocular opacity, posing challenges to investigators who may be combining behavioural parameters of metabolic syndrome and diabetes in their investigations. From these previous studies, it is clear that the literature is highly variable, and is conflicting. This includes information on the preparation and administration of STZ, the time to onset and severity of diabetes, as well as morbidity and mortality of animals. High mortality rates of animals only become apparent once the results have been critically evaluated, and in spite of initially large cohort sizes, results of these studies may be misleading.

**Animal models are often used to investigate disease processes**

**Transgenic and polygenic mouse models**

Despite what may appear to be obvious advantages of genetic mouse models such as the db/db model for metabolic syndrome or diabetes induction in humans, it is still difficult to discern whether IR precedes or is secondary to the development of obesity. With mouse models, it would appear that profound obesity predisposes animals to diabetes. This mechanism is primarily due to the leptin gene, or the leptin receptor mutation, which results in subsequent hyperphagia. Humans simply do not develop either metabolic syndrome or diabetes from over-eating. Reports indicate that the db/db mouse becomes hyperinsulinaemic within two weeks of age with obesity developing by three to four weeks and subsequent β-cell failure from week’s four to eight. Other models used include the fat mouse which has a mutation-specific gene to carboxypeptidase E which is involved in insulin metabolism, the rarely used tubby mouse, and the spontaneous hypertensive rat. A significant limitation is that these models induce DM by failure to adequately increase β-cell mass in response to obesity-induced IR. This fails to demonstrate the similarities for islet cell pathology typical of T2DM (islet amyloid) seen in humans. Genetic models such as C57BL/6 appear more susceptible to obesity and diabetes, whereas mice such as the dilute, brown non-agouti appear to manifest islet cell failure quicker than other strains. Furthermore, in practical terms, the use of obese rats or genetically modified obese mice pose numerous challenges. These challenges include reliabilities associated with performing neurological investigations, possibly limiting the choice of parameters tested, as well as sourcing appropriate equipment that would allow for the free and unrestricted movement of animals.

**Dietary animal models**

Dietary models have become fairly popular as they better represent obesity and diabetes as seen in humans. A number of dietary models and versions exist – be it high-fat; high-fat plus cholesterol; high-fat and carbohydrate; or high sucrose; or even high fructose feeding regimes. The high fat model, known as the diet-induced obese model, is a common model where animals derive more than 60% of their calories from fat. These animals become hyperphagic quickly, with a resultant over-consumption of calories, display a reduction in thermogenesis, reduced energy expenditure, and become seemingly ‘lazy’. The golden Syrian hamster shares a number of similarities to humans in lipid metabolism and physiology. These rodents are a fairly successful model of IR and metabolic syndrome on a high fructose diet. Dogs fed high fructose diets display pre-diabetic parameters, with almost all models that use fructose having some success when investigating diabetic conditions. There are a few exceptions to this sensitivity, with certain mouse models resistant to fructose due to mutations in the sterol regulatory element-binding protein-1 gene. This gene regulates lipid and cholesterol production as well as sterol levels at cellular level. These sensitivities to fructose may be further explained. This is due to the inability of fructose to stimulate the production of leptin hormone, which in part, plays a role in the regulation of energy intake and expenditure. Fructose does, however, cause de novo lipogenesis and triglyceride synthesis.
There is extensive literature that investigates ‘sugar’ under a number of aliases within numerous contexts.54-57 These aliases, which are often used interchangeably, include, but are not limited to, sucrose, glucose (also known as dextrose), maltodextrin, fructose, free sugar and galactose, as well as high fructose corn syrup. High fructose corn syrup contains between 55% and 90% fructose and corn starch. The use of sucrose has become increasingly popular as a model of sucrose-induced IR and hypertriglyceridaemia54,55 as metabolic dysregulation is reported to occur in one to two weeks of animal feeding.56,57 Almost all of these ‘sugars’ used in the food industry are derived from various sources, and are all metabolised differently. The wide range differences cited on fructose in particular, as well as inadequately detailed methodologies make it difficult to reliably replicate study conditions and compare results across studies, making it problematic to translate to humans.

Exacerbating the above challenges, most animal studies investigating the effects of diet do not use a nutrient and energy matched control feed, but rather ‘standard chow’ which differs in composition from different suppliers. Furthermore, the thrust of most small animal studies investigating the effects of high sucrose diets on metabolic alterations have been conducted in males.58-60 This makes it difficult to draw conclusions about the effects of high ‘sugar’ intakes on females, unwittingly biasing research and making it difficult to understand or translate findings to humans.

Prenatal dietary manipulations (foetal programming) are used occasionally, with high intakes of protein diets reported to reduce β-cell proliferation and reduced islet size.18 These models may offer insights into genetic predispositions, epigenetic modifications, and the role of nutrigenomics in T2DM onset.61 However, limited literature sources caution about generational animal insensitivity when attempting to induce these changes.10,40

Based on the above, it is important to take cognisance that laboratory animals, particularly rat and human metabolic processes, do not always run in parallel. Rats and humans process fats and cholesterols differently attributable to the fact that rats have a higher activity of the liver enzyme 5-desaturase.62 This increased enzyme activity in rats alters the chemical structure of fats which affects the metabolism of plasma lipoproteins. Compounding this, rats have no gallbladder, hence bile manufactured under the influence of muricholic acid is directly secreted into the intestine, rapidly eliminating cholesterol.63 For this reason, rats are resistant to changes in serum cholesterol levels as well as dietary induced arterial plaques.64,65 In contrast, humans conventionally route bile via the entero-hepatic circulation and do not manufacture muricholic acid and simply need to ingest fats to cause plaques. Furthermore, bile aids in the control of arterial plaques.

Another significant difference between rats and humans is the ability of the rat (and most mammalian animal species) to synthesise Vitamin C in the liver from glucose.69 Humans are unable to synthesise Vitamin C due to the absence of L-gulono oxidase and D-glucuronol reductase enzymes. These enzymes respectively play roles in neutralising free radicals, with D-glucuronol reductase largely responsible for changing the functions of blood, skin and joints.69

Compounding these anomalies, is the difficulty to induce muscle IR in rodents,70 particularly rats, as the muscle fibre (sub) types, by nature of the animal, are different in ratio to that of humans. Factoring all these differences and using metabolic syndrome and diabetes as examples of the complexity of disease processes, it is evident that the validity of animal models used to study human disease is not without controversy.

Animal ethics committees within higher education institutions (HEI’S) in South Africa are guided by the South African Veterinary Council for the use of animals in research. Stringent and robust processes under Section 21 of the Veterinary and Para-veterinary Act 19 of 1982 of the South African Veterinary Council (SAVC) now require an investigator to formally register with the SAVC. This authorisation carries a financial burden to the researcher, with the caveat that successful authorisation (and registration) is finite i.e. has a time limit. Under this scheme falls the additional responsibilities of adopting the 3 R principles (replace, reduce and refine). These constraints, which may include ‘getting the most from our model’ leans to collaborative research across scientific disciplines, or multi-disciplinary teams using a single model to investigate numerous aspects of a disease process. In simple terms, this may mean, responsibly harvest and maximise as much information as possible, from a single model, across disciplines with a stringent ethics approval issued for the primary investigator.

CONCLUSIONS
Animal models are not universally favourable, are not without risk, and ‘one size does not fit all’.25,62-67,71 In spite of almost 40 years use of STZ in small animal models, standardisation on DM induction with this modality is yet to be achieved by scientific communities.46 This makes reproducing a study elsewhere extremely difficult and comparing results across studies, challenging. Furthermore, the use of both STZ and alloxan as agents to induce either diabetes or metabolic syndrome in animals does not mimic the aetiology of the human disease process. This also highlights a few critical discrepancies relevant to metabolic syndrome and diabetes. It illustrates just how confusing it may become when selecting an appropriate animal model to mimic the human condition.

The primary focus of all biomedical research remains that of optimising benefit, as well as ensuring that the results produced are (or can be) translated into a clinical setting.72 It is important to avoid oversimplifications of any disease process under investigation. It is also extremely important that a researcher weigh the benefits of the research against the burdens. Achieving this balance of cost and benefit is not easy, as the two are not measured in the same terms. Animal protection should be a permanent consideration, and while the objective is to enhance medical care, the two objectives can often be antagonistic.73 However, the results should still be anchored in quality science. Ultimately, results generated from research are intended to enhance knowledge in order to benefit patient care.71 While neutral or negative results from animal studies are for obvious reasons more likely to remain unpublished, a central data base, freely accessible to all researchers should be encouraged. Factors such as
REFERENCES


FURTHER REFERENCES ARE AVAILABLE ON REQUEST.