An efficacy study for an an anti-arthritic drug

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As Chairman of an Animal Ethics Committee, I consider that there is a major requirement to ensure that animal welfare is given priority without the sacrifice of good science. As a committee responsible for overseeing regulatory testing for the pharmaceutical industries, both human and veterinary, we note that most of the test outlines are already developed and have been so for decades.

One particular study that was presented to us was an efficacy study for an anti-arthritic drug. First question to be raised by the committee was, "Why?" Why is it necessary to have another anti-arthritic product? Why is this one different? But more importantly, "Why does the model require a positive untreated control group, even though this is stipulated in the regulatory model outline? The justification for this new treatment was that unlike current treatments, it was believed that it would not only arrest the arthritis but would repair and regenerate the joint. Clearly a marked improvement on existing products. Then it was discussed with the sponsor and it was agreed that we should approach the Regulatory Agency and see if we could eliminate this untreated control group. After all, the study director and histopathologists working on this study would know what a progressive arthritis looked like. Since a treatment group was included with an established licensed treatment, we had a measure of treatment to be achieved in the sense that the new product being tested would have to either meet or exceed the established treatment. If it couldn't do this, it wasn't an improvement on existing drugs and therefore wasn't needed on the market. The argument was made and we had agreement that a non-treated group was not necessary and was not in the best interest of animal welfare. Any time a change can be made to an established regulatory model of testing, it is a tremendous achievement.

But the story doesn't end there. A long time ago, the regulatory agencies lowered the number of animals required per group as part of their responsibility to Reduce and Refine. The number now is six per group—enough points of reference to establish a statistical significance but far below the number required when a power analysis is performed in order to achieve an 80% level of efficacy, the usual requirement for many drugs. One of the members of the committee, coming from a research background argued that he could not approve the study because he didn't feel there were sufficient animals on test. Since the committee works by unanimous consensus, we were unable to approve the study initially. As Chairman, it was my responsibility to provide the member with enough supportive information to show that the test has been effective using such numbers for the 35 years it had been in existence and that approving an increase in numbers was not only unnecessary but totally against the concept of the Three Rs that we promoted. Unfortunately, to those of us familiar with statistics, it is a science that is considered black and white, no shades of grey. That member of the committee could not let go of the fact that the Power Analysis called for over double the number of animals on the study. He would not agree, with the lower numbers no matter what evidence was provided to him suggesting otherwise. Several meetings later, the member contacted me to say that he was resigning as he did not feel he was given sufficient credit for his contributions. Reluctantly I accepted. But the underlying statement to this entire study in the realm of "Certainly not what I expected" is that nothing should be considered immovable—not Regulatory Agencies, not the science of Statistics. Having a good argument can move a mountain. As an Animal Ethics Committee we are dedicated to the Three Rs and any reversal from those principles would not only be to the detriment of Animal Welfare but to Science itself.

Using animal models to study human disease states

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This presentation discussed some of the challenges and successes when using animal models of exercise and diet to study human disease states. The top three public health issues over the coming decades will be the ageing population, obesity and type II diabetes. These conditions (and potential treatments) naturally take many years to develop in humans and can be mimicked in animals, usually rodents. Dr Harrison's research was specifically on the impact of diet (olive and macadamia oil rich) and endurance exercise on heart structure and function. He discussed the challenges of training rats to run on a treadmill (e.g., "Speedy-G", a rat that voluntarily ran backwards!) and the value of rewards-based methods and the patience/understanding needed from investigators for reliable results. Dieting in animals also created some unexpected challenges such as behavioural changes when rats maintain a lean and healthy body weight in a standard housing environment. The talk concluded with an outline of the array of critical factors involved in animal care, welfare and ethics from the animal, animal house and scientist standpoints.

Views expressed are those of Dr Glenn Harrison and not necessarily of Griffith University.

Animal ethics committees for schools

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In 2003, the Department of Education and Training (DET) established the SAEC consistent with the requirements of the *State's Animal Welfare Act (2002)* (the Act) and the *Code of practice for the* use *of animals for scientific purposes* (NHMRC 1997) (the Code).

An agreement was reached that the SAEC would also serve as the Animal Ethics Committee for the Association of Independent Schools of WA (AISWA) and the Catholic Education Office (CEO), undertaking the same role for them as it does with the Department.

In 2007, four of the nine SAEC meetings were held on an educational site. The meetings were followed by a conducted site tour. The site visits served two purposes.

They increased SAEC members' understanding of the breadth and depth of the educational program and the nature and quality of the facilities that support the program and they increased host organisations' knowledge of the SAEC.

In 2007–2008, professional development workshops for teachers and laboratory technicians were held at various locations around the State by representatives from the SAEC to assist them to:

- gain a greater understanding of sound animal welfare practices,
- explore teaching strategies that may be used when dealing with ethical, welfare and legal issues related to the use of animals in schools and agriculture
- become familiar with a variety of online resources suitable for use in teaching

Modelling Crohn's Disease

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Inflammatory bowel disease (IBD) is as common in Canterbury, New Zealand as anywhere in the world, with the incidence and prevalence of Crohn's disease (CD) amongst the highest ever reported. CD usually presents in early adulthood and manifests as severe intestinal inflammation. There is no cure and the cause is poorly understood. The rate of increase in CD cases indicates the involvement of factors in addition to host genetic susceptibility. Experiments with different mouse models suggest that gut bacteria may play a causal role but a specific bacterium has not been identified.

One bacterial species that stands out as a plausible single candidate in the aetiology of CD is *Mycobacterium avium* subsp. *paratuberculosis* (MAP). MAP is the causative agent of Johne's disease in ruminant animals, with similar clinical symptoms and pathology to CD in humans. Studies report increased molecular

and microbiological evidence of MAP in CD patients. However, whether this simply reflects translocation of gut flora through a leaky intestinal epithelial barrier or whether this organism has a role in the aetiology of CD is still unknown.

Our aim was to use interleukin (IL)-10 knockout model, based on evidence that these mice spontaneously develop a Crohn's-like colitis, to determine whether introduction of MAP infection would enhance the development of this colitis. The animals were assessed weekly for 20 weeks and any sign of disease activity scored using an index developed for assessing CD in humans. We found no significant difference in disease activity and/or histological scores in naïve versus MAP-challenged mice. This suggests that the development of Crohn's like disease in this mouse model requires more than a genetic susceptibility and/or exposure to MAP.

However, despite being slow to develop colitis, four mice developed neoplasms in the caecum. The cancers, which were consistent with an invasive adenocarcinoma, were associated with severe localised inflammation. These results suggest we have a potential model of inflammation-induced colon cancer.

Zebrafish: Human disease modelling and treatment strategies

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Introduction

The modelling species

The zebrafish is an established model for studying developmental vertebrate biology that also offers opportunities for modelling human diseases. The advantages of the zebrafish include the following: optical clarity of zebrafish embryos and larvae, which allows for real-time *in vivo* visualisation of vertebrate development (Eisen 1996); rapid embryonic development such that after the first cleavage, the blastomeres divide at about 15-minute intervals with most organs in place by day 6 post-fertilisation (Kimmel et al. 1995); high fecundity, with large numbers of embryos being produced at a significantly lower cost compared to mammals; and a high degree of coding sequence conservation between human and zebrafish genomes. The zebrafish combines many of the invertebrate features of scale and throughput, coupled with vertebrate anatomical and physiological complexity. The above attributes position the zebrafish as a suitable whole-animal vertebrate for compound screens in order to identify lead therapeutic molecules. Critically, the zebrafish is permeable to many small molecules (Peterson et al. 2000). Compound screening of chemical libraries can be performed using microtitre plates that contain zebrafish embryos in the presence of a range of concentrations of defined or complex molecules.

The human disease

Huntington disease (HD) is an inherited autosomal dominant neurodegenerative disorder that is caused by an expansion of a CAG trinucleotide repeat within exon 1 of the *HD* gene. Expansion of the CAG repeat leads to an abnormally long polyglutamine tract (polyQ) in the amino-terminus of the huntingtin (Htt) protein. The mutation leads to a progressive selective degeneration of the GABAergic medium-sized spiny neurons in the striatum (Reiner et al. 1988; Vonsattel & DiFiglia 1998), and the pyramidal neurons in the cortex (Cudkowicz & Kowall, 1990; Macdonald & Halliday 2002). Mutant Htt is sequestered in cytoplasmic and nuclear inclusions within affected pyramidal neurons (Davies et al. 1997; DiFiglia et al. 1997). The appearance of these protein aggregates is characteristic of the disease (Scherzinger et al. 1997).

The challenge

There are no known treatments for HD, but recent reports have identified two small molecules that can reduce aggregate formation of mutant Htt in several species used for modelling. In a large scale *in vitro* screen of over 5000 naturally occurring substances, Epigallocatechin-3-gallate (EGCG) inhibited the aggregation of mutant Htt protein (Kuriyama et al. 2006). EGCG is a naturally occurring molecule found in green tea that has been proposed to offer health benefits through antioxidative activity (Yang et al.

2002). The *in vivo* effects of EGCG were assessed in yeast and fly models of HD and inhibition of disease phenotypes was found in each model.

In addition, trehalose appears to be an effective inhibitor of polyglutamine aggregation (Tanaka et al. 2004). Trehalose is a non-reducing disaccharide that provides protection from desiccation, dehydration and oxidation by maintaining protein conformation and preventing protein aggregation of unfolded proteins by direct protein-small molecule interactions (Richards et al. 2002; Singer & Lindquist 1998a; 1998b). The oral administration of 2% trehalose to a mouse model of HD led to neuroprotective properties such as reducing striatal atrophy and intranuclear huntington-ubiquitin inclusions, improving motor function and increasing life expectancy (Tanaka et al. 2004).

Against the above background, we were interested in establishing a transient model of HD in the zebrafish and testing the efficacy of EGCG and trehalose in reducing mutant Htt protein aggregation.

Research aim and objectives

The over-arching aim of the research was to achieve the direct visualisation of a truncated mutant Htt protein fused to the green fluorescent protein (GFP) reporter, and to undertake real-time *in viv*o assessment of EGCG and trehalose on aggregate formation. This aim involved the following three objectives:

Objective 1: To achieve zebrafish embryos expressing fluorescent polyglutamine aggregates. This objective involved the microinjection of zebrafish embryos with DNA constructs carrying either the CMV or EF1α promoter driving the expression of an in-frame fusion of exon 1 of the *HD* gene with two different sized (CAG) repeats, and the eGFP gene.

Objective 2: To assess the effect of EGCG and trehalose on zebrafish embryos. Uninjected zebrafish embryos were treated with a range of concentrations of EGCG and trehalose in order to determine the maximum tolerated concentration of both small molecules.

Objective 3: To treat zebrafish embryos expressing fluorescent polyglutamine aggregates with EGCG and trehalose. This objective used the optimised microinjection protocol identified in objective 1, together with the maximum tolerated concentration of EGCG and trehalose from objective 2. The treated embryos were examined with regard to survival, morphology and fluorescent aggregate formation.

Conclusions

1. The transient modelling work described here reproduced an important feature of HD, i.e. the formation of polyglutamine protein aggregates.
2. The toxicity assays were found to be robust and simple to set up, and involved only the use of routine light microscopy without the need for microinjection.
3. The maximum tolerated concentration of EGCG and trehalose for the purposes of evaluating their efficacy in inhibiting polyglutamine aggregation were found to be 100 μM and 2% (w/v), respectively.
4. EGCG and trehalose did not appear to inhibit polyglutamine aggregation in the zebrafish HD model. These results were unexpected given the literature supporting the effect of these small molecules on polyglutamine aggregate formation.
5. There was considerable variability in the expression of polyglutamine protein and aggregate formation. This underlying variability may explain why no statistically significant reduction in the level of aggregate formation was detected.

Future work

The establishment of stable transgenic zebrafish models of HD would be expected to enable better disease modelling to be realised, and hence reduce variability between zebrafish in terms of Htt protein expression. In this context, recent advances in gene delivery systems in zebrafish genetics have significantly improved the efficiency and stability of establishing transgenic fish lines (reviewed by Kawakami 2005). Interestingly, transgenic zebrafish lines could be used in a novel manner in understanding the length of time that is required for mutant Htt protein expression to effect a pathophysiological response. This type of research would involve the use of regulated promoters driving the expression of mutant Htt.

Overall, the above work did not fulfil our expectations but has led to a refinement of our method in the hope of developing a better human disease model

that will allow high throughput testing of possible therapeutic molecules.

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