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Experimental Design: Balancing Statistics with Biology

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Both scientists and animal ethics committee (AEC) members believe that it is essential to plan experiments with a view to ensuring that the data obtained will be statistically significant, as this is seen as one important measure of scientific validity. In situations where those experiments will rely on the use of animals, this imperative goes even deeper as experimental design and statistical validity of the resulting data are also seen as key indicators that the number of animals proposed is appropriate. This principle is of course, inextricably linked to the 3Rs (Replacement, Reduction and Refinement) that are clearly spelled out in the Code and ingrained in the ethos of AEC deliberations that the number of animals to be used in any experiment must fulfil the Goldilocks principles of not too many and not too few. The number of animals in any experiment needs to be "just right". This means that using more animals than would be required to gain a statistically significant outcome would contribute very little (if anything) to the experiment and just waste the lives of those additional animals, while the use of too few animals would mean that the experiment would not produce any significant or publishable data and so those animals would also have been wasted. Not surprisingly therefore, the question of experimental group size and the number of animals to be used in any scientific work can often be a point of discussion and potentially some tension – both within an AEC and also between the AEC and the applicants it serves.

While there are a number of factors that can be used to guide the deliberations of an AEC when it comes to the number of animals to approve, clearly one of the more popular approaches is to ensure that applicants include a power calculation (or similar) in their application as a way of justifying the number of animals they have requested. Such calculations serve to reinforce the idea that a number of factors must be considered as a part of the process of designing experiments and in many cases provide an excellent indication of the most appropriate group size, but clearly the outcome of such calculations can be influenced by

certain factors and may not always be the best way to assess the optimal number of animals to use.

One example of how experimental design can profoundly influence the number of animals required to complete the work is the issue of confidence. Confidence is an important variable in power calculations and essentially reflects the level of surety one has in the experiment delivering the correct answer. Ideally, we would all like to believe that we could reach 100% confidence in the results obtained from every experiment, but unfortunately this is not realistic as nothing will ever be absolutely certain in the biological world. From both an ethical and a mathematical perspective, certainty in the outcome would require an almost infinite number of animals in each experiment. At the other end of the spectrum, if you are working towards minimising the number of animals required by only striving for a confidence level of 50% or less in the data obtained from your work, then there is absolutely no point in doing the experiments as you would get exactly the same information from tossing a coin. So clearly there must be some compromise between these two extremes. While most researchers work around the 80 – 85 % confidence level, which does fulfil both aims of having confidence in the outcome of their work and ensuring that the work can practically be completed, this figure will vary depending on the nature of the work being done.

Another major factor that influences the number of animals required to complete a project is the magnitude of the expected response when compared to what is seen in control animals. So in cases where experimental outcomes are clearly different (i.e. the outcome being one where you can observe a response versus one where there essentially is no response), the number of animals required in each experimental group will be much smaller than you would need if the experimental outcomes are very similar between the test and control groups. All this means that experimental design can play an important role here as well because the bigger the response that is seen, the fewer animals will need to be used. Of course, this may also have some ethical implications as a 'big response' may in some cases come at a welfare cost to the animals – but we will come back to this idea a bit later on. This does however help to raise the concept of 'biological significance', where the differences in results obtained between experimental groups can clearly be biologically significant, for example eliciting a protective immune response versus no immunity, or life versus death, even in cases where the numbers involved are too small to reach statistical significance.

To put all this into context, the role of statistics is to provide validation to the experimental conclusions. It is easy for statistics to become an end within themselves and this should not be the case. When factoring the statistical analysis into the design of your experiments, the starting point needs to be - What is the hypothesis being tested and therefore what level of experimentation and statistical analysis is required to achieve that endpoint? This is of particular importance when viewing the hypothesis from an AEC perspective, particularly in projects where more than one hypothesis might be tested as there may be different degrees of confidence, for each one, so the statistical parameters may also vary.

There are other ways to improve experimental design with a view to improving the validity of the data obtained and these include ideas such as the randomisation of animals to each group and the blinding of scientists to treatments received by the animals they are observing and in which they will potentially be measuring a response. The concept of randomisation is (at least from a statistical point of view) fairly simple in that it is merely trying to ensure that animals will be randomly assigned to either a treatment group or a control group, etc. Of course in practice, this needs to be slightly more complex than just grabbing mice from a box and placing them into groups by alternating between them. While this is a tactic that is often employed, it is more often a selection based on individual personality (e.g. bold or timid), speed and agility (the ability of mice to evade the 'hand of selection') or other such traits, so it is not truly random selection. This does not mean that something like selection order cannot be used to randomise animals into groups, it just means that you would need to use a random number generator (or alike) to determine which animals were assigned to each group. So, for example, fish or mice (in section order) 3, 4, 9, 13 & 17 might go to group A, while animals 1, 2, 6, 15 & 20 go to group B, numbers 5, 7, 10, 18 & 19 are assigned to group C and animals 8, 11, 12, 14 & 16 are placed in group D. The important difference is that the random number table has removed any bias that might be associated with individual traits associated with any of the animals being used.

When considered from a purely mathematical (or statistical) perspective, the random assignment of animals to each group in an experiment is ideal and will significantly improve the design of an experiment and the reliability of the data obtained. However from a biological perspective, there may be some additional and complicating factors that have to be considered. For example, if the proposed work involves the use of male mice, there could be some significant problems with mice fighting if they are reassigned to different cage groups and feel the need to re-establish a 'pecking order' within the new group. As experienced animal care staff will tell you, even the simple act of splitting a group of some strains of male mice into two groups after they have been raised together since weaning

will result in serious fighting while they re-establish a hierarchy within each smaller group. The battles that follow within each sub-group of mice can result in significant scratches, bites and other injuries that will affect the stress levels for individual animals, potentially result in infections and generally impact on the health and welfare status of the animals in your study. In other words, the value of any data generated may be regarded as questionable. So even though the idea of randomising subjects between experimental groups and control groups is an excellent way to strengthen the design of an experiment from a statistical perspective, there may be biologically important reasons that would make this impractical. So yet again, we find ourselves balancing out the differences between statistical significance and biological significance – albeit in a very different way.

One area where I suspect some significant improvement to the design of experiments may be simple to achieve is through the introduction of experimental 'blinding'. In this context, 'blinding' simply means designing an experiment in such a way that ensures the person measuring the results either during or at the end of each experiment, does not know what (if any) treatment has been administered to each animal. This should include whether the animal is part of a treatment group or a control group. It is amazing to see how even knowledge of whether an animal or assay well in cell a culture plate is associated with a test or control group can result in even a very subtle or minor bias that can influence the outcome of an experiment or trial, so ensuring that those treating animals are not involved with the reading or interpretation of data obtained from those animals can be a really important step towards improving the quality of the data generated and it costs virtually nothing.

Even in situations where an approval may include provision for 'blinding' of investigators, there may be occasions where it is not being done in a way that is truly effective and in many of these cases this may result from limitations associated with small staff numbers (in some cases potentially only a single animal technician doing the work) and this would mean that additional thought might be required by both the investigators and the AEC to see if there is a simple way to improve the effectiveness of blinding the person charged with determining the results of the study. This may be as simple as getting someone outside the project team to blind samples between the time of harvest and subsequent measurement. In other cases, more carefully planned steps may be required, but would definitely be worth the effort.

So yes, we must be very mindful of appropriate statistical design through the use of measures like the randomization of subjects, ensuring appropriate sample size and blinding of samples, but these factors always need to be considered in the context of the biological and welfare requirements of the animals as well.

Vale:- Dr Yvette Chen

It is with great sadness that we acknowledge the passing of Yvette Man Man Chen who lost her long battle with cancer on Tuesday 17th of March, 2015.

Yvette graduated from the Melbourne University School of Veterinary Science in 1998 and worked in clinical practice until her interest in research resulted in her undertaking a Ph.D. After completing her Ph.D., Yvette moved to the UK and worked as a Veterinarian in the Pharmaceutical industry for around five years and also managed to complete a Certificate in Laboratory Animal Science through the Royal College of Veterinary Surgeons, which set her on course for her future career back in Australia.

When Yvette moved back to Melbourne in 2008, she worked briefly as Principal Veterinary Officer at the Bureau of Animal Welfare in Victoria before taking on the role of Animal Welfare Officer at the University of Melbourne. Though small in stature, Yvette grew to be a giant in this, her chosen career and her boundless energy saw her battle through her first diagnosis and the treatments that followed.

Once she had recovered and returned to work, Yvette continued to power ahead in her role as AWO with typical energy and enthusiasm as well as taking on the Presidency of ANZLAA, serving as a member of the NHMRC Animal Welfare Committee, assisting with the revision of the Code and becoming a Mum – which Yvette considered her greatest achievement.

News that follow-up scans had detected a recurrence of the tumour was devastating for Yvette's friends and colleagues as well as her family, but she faced the battle with typical resolve and good humour and maintained her professional interests as much as her health allowed. So it was always encouraging to see an email from Yvette with advice posted in response to questions that had been circulated.

Yvette passed away surrounded by family and friends and on behalf of everyone associated with ANZCCART, we would like to offer our deepest condolences to all her friends and family, particularly to her husband Brendan and her son Finnie who were the centre of her universe.

Dr David Bayvel QSO

As this edition of ANZCCART News was being finalised, we were greatly saddened to learn that Dr David Bayvel (Honorary Life Member) passed away as a result of an illness on Tuesday 7th of April, 2015 in Waikanoe, New Zealand. A more fitting tribute to David will be included in a subsequent edition.

Letter to the Editor

The following comment was submitted in response to the article we published late last year, "Can we further refine humane killing with CO_2?" The original article can be read here.

I would like to respectfully voice my objection to the title of your recent article on the use of CO_2 for killing animals that was published in *ANZCCART News Vol 27 No. 4 (2014): "Can we further refine humane killing with CO_2?"* Below, I will provide an account of the reasoning underlying my objection.

This title strongly implies that the use of CO_2 to kill (or stun) animals can be considered humane. As someone who is active in this field of research, I am of the strong opinion that there is currently insufficient scientific evidence to support this notion. In fact, all scientific evidence to date supports the notion that the use of hypercapnic gases to stun or kill animals is inhumane, i.e. it causes significant aversion in all mammalian species tested to date, regardless of the method of induction (gradual versus full immersion). The exception to this may be some species of poultry which don't appear to show behavioural aversion; however, our current understanding of respiratory control and cognitive processing of 'noxious' sensory inputs in birds is very limited.

The features of hypercapnic gas inhalation that may be aversive to mammals include pain due to mucosal/ocular acidification – this seems to be the main focus of most reports on the 'humaneness of CO_2 killing' (e.g. see excerpt from AVMA Guidelines for Euthanasia below) and the absence of such pain before loss of consciousness is most often the basis of conclusions that its use is humane. However, hypercapnic gases are also likely to cause unpleasant sensations of breathlessness (sensations of respiratory work/effort and/or air hunger). Air hunger is now understood to be one of the most strongly unpleasant and most immediately compelling sensations experienced by mammals because of its critical survival value to the animal ([1]Beausoleil and Mellor, 2015). We do not yet understand the potential for breathlessness, particularly air hunger, to occur during CO_2/hypoxia killing in animal species, nor do we have a way to measure the occurrence or associated aversiveness of such experiences. In addition to breathlessness, there is also, potentially, an independent sensation of choking/suffocation/panic, the physiological basis of which is not yet well understood but which is a consistently reported phenomenon in humans inhaling even relatively low CO_2 tensions (e.g. 8%). In addition, cerebral vasodilation often leads to sensations of headache, dizziness and nausea in human subjects well before pCO_2 reaches levels required for narcosis or loss of consciousness. There are likely to be significant species differences in such experiences of hypercapnic gas inhalation; species differences have been shown for aversion to hypoxic gas inhalation (rodents are far more sensitive than humans, for example).

The AVMA Guidelines for Euthanasia 2013 consider CO_2 'Acceptable With Conditions' for euthanasia – however, further reading reveals that the main condition is probably not achievable in practice for most species at this time: *"…acceptable with conditions for euthanasia in those species where aversion or distress can be minimized. Carbon dioxide exposure using a gradual fill method is less likely to cause pain due to nociceptor activation by carbonic acid prior to onset of unconsciousness."*

It is my opinion that until we have clear scientific evidence that CO_2 stunning/killing can be administered in such a way as to minimise or ameliorate ALL of these potentially unpleasant experiences, we should not be using it, nor should we be making any suggestion that it is humane and can thus be refined to make it 'more humane'. Such suggestions may lead scientists, animal carers and policy makers to believe that CO_2 can currently be used to bring about a humane death, leading to the continued use of this method for killing many thousands of animals each year. I (and others) are very uncomfortable with the continued use of carbon dioxide to kill mammals until we better understand the welfare implications of its use.

Many thanks for your time in considering my position.

Ngaio Beausoleil BSc, PGCert(Sci), PhD
Deputy Director,
Animal Welfare Science and Bioethics Centre
Massey University

[1]Beausoleil, N.J. and Mellor, D.J. 2015. Introducing breathlessness as a significant animal welfare issue. *New Zealand Veterinary Journal (Animal Welfare Special Issue)* 63(1): 44-51. DOI:10.1080/00480169.2014.940410

2015 ANZCCART Conference
21 to 23 July
Gold Coast, Australia

ANZCCART would like to announce that the Call for Abstracts is now open.

Closing date is Monday 1 June

Conference Registration is now open.

Early Bird Registrations close Friday 22 May

For further information and registration visit:

http://www.adelaide.edu.au/ANZCCART/

ANZCCART AEC
Member of the Year Award
for 2015

ANZCCART is calling for nominations for the 2015 AEC Member of the Year Award and welcomes nominations for outstanding AEC members who are currently serving on one or more AECs in Australia and New Zealand.

The prize includes an all-expenses paid trip to the annual ANZCCART Conference for the winner.

Nominations close on Friday 8 May 2015.

Please refer to the ANZCCART website for full terms and conditions of the award at:

http://www.adelaide.edu.au/ANZCCART/awards/

Paul Flecknell Workshop

Assessment, Prevention and Alleviation of Pain and Distress in Laboratory Animals

This workshop will provide an up-to-date review of the assessment, prevention and alleviation of pain and distress in laboratory animals. The workshop will be of interest to veterinarians, animal technicians, research scientists, and members of ethics committees. Seminars will be extensively illustrated with video material and ample time provided for discussion.

20 to 22 April 2015
Newcastle University, Newcastle Upon Tyne,
United Kingdom

More information can be found here.

Animal Welfare Officer Position
Flinders University
(Ref 15120)

The Animal Welfare Officer provides specialist expertise in the management of animal welfare related to research and teaching conducted by University staff and students. The incumbent will work closely with the University's Animal Welfare Committee, animal facility staff and the Research Services Office, to ensure that the use of animals in research and teaching is carried out according to the highest standards of animal welfare, and complies with relevant State legislation and national guidelines. The incumbent will possess a veterinary qualification and experience with a range of animal species, and will bring a proactive approach and excellent interpersonal skills to the role.

Available on a continuing, part-time (0.5 FTE) basis. Salary (HE09): $98,346 to $105,369 pa (full-time), plus 17% employer superannuation.

Applications close: 11.00am, Monday 27 April 2015.

Full details can be found here:
http://www.flinders.edu.au/employment/vacancies/general.cfm
Applications will only be accepted via the website, as described here:
http://www.flinders.edu.au/employment

Recent Articles of Interest:

Cancer patients testing drugs on mouse 'avatars'

Cancer patients are using mice to test drugs and personalise their own treatment with the hope of curing their disease. Private laboratories breed the mice and patients pay to have a tumour sample banked and for drugs to be tested on mice which have been implanted with parts of their tumour.

Seventy patients whose outcomes from treatment were already known, were studied to see how well the mice performed. Results showed that for 70% of the time, tests in the mice suggested that something had helped the patients and if something had not worked in the mice it almost never worked in a patient.

However, mice do have some drawbacks such as the graft is only implanted under the skin and not in organs where the cancer may be and also their immune systems are highly impaired and differ to humans.

While it might be reasonable to question the scientific validity of such studies, patients though are more confident and reassured when they see their mice responding to the drugs and as one patient said she didn't see it as animal abuse but as testing to try to save her life.
https://news.yahoo.com/cancer-patients-testing-drugs-mouse-avatars-172416007.html

Radio-controlled mouse hints at new diabetes treatment

Genetically altered mice have responded to a radio signal by releasing insulin, which lowers blood sugar. This response relies on the presence of iron particles in the body, allowing energy from radio waves with the right frequency to be absorbed thus changing cell behaviour.

This approach has shown that it is possible to regulate processes within the body remotely and could potentially be tailored to treat other diseases too. Although it is still a long way from being used therapeutically in people, researchers believe that one day it just might allow people with type 1 diabetes to use mobile devices such as their mobile phone to control their disease or to lessen jerky movements in sufferers of Parkinson's disease.
http://www.newscientist.com/article/dn26701-radiocontrolled-mouse-hints-at-new-diabetes-treatment.html#.VJC23f4cTIW

Ferret mutation makes them ideal flu model

For years, researchers have known that ferrets can be susceptible to human influenza A virus but the reason was unknown.

A collaborative team of researchers from Australian Universities have discovered that ferrets share a mutation previously thought to be unique to humans, leading to a new approach in dealing with human diseases. Read the full article at:
http://www.alnmag.com/news/2014/12/ferret-mutation-makes-them-ideal-flu-model?et_cid=4331955&et_rid=497549351&type=cta

Tickling rats reduces stress of injections

The findings from a new study at Washington State University could have implications for laboratory animal welfare.

When giving injections researchers have been tickling rats in a way which mimics their social play behaviour and have found that the rats show less stress and are easier to handle.

The study can be used to help refine laboratory animal care standards and improve the quality and validity of research data, as well as improving animal welfare.
http://www.alnmag.com/news/2015/02/tickling-rats-reduces-stress-injections?et_cid=4412495&et_rid=497549351&type=headline

UK Funders demand strong statistics for animal studies

It would appear that the need to consider possible improvements in experimental design is an international issue with a recent study in UK raising concerns about the number of underpowered experiments being carried out. That study has highlighted the need to ensure that using the right number of animals is an essential component for both scientific validity and ethical assessment of any work.

As a result of the study reported, UK funding bodies will now require clear and appropriate justification of animal numbers as a part of their application process.
http://www.nature.com/news/uk-funders-demand-strong-statistics-for-animal-studies-1.17318?WT.ec_id=NATURE-20150416

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The opinions expressed in *ANZCCART NEWS* are not necessarily those held by ANZCCART.

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