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Underreporting of the three Rs deployment that occurs during the planning of protocols that precedes their submission to animal ethics committees

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Abstract
Application of the Three Rs occurs during two stages before an investigation begins: (1) during the preliminary planning and preparation of the applications to undertake specific projects, before they are forwarded to the AEC; and (2) by the AEC after it receives applications from investigators. Examples of Three Rs applications are documented in the finally approved protocol once the AEC has completed its deliberations. However, their innovative character and reference to the less acceptable procedures they replace are often not mentioned in the approved AEC applications. Indeed, the AEC may have no knowledge of what innovative approaches have been applied in Three Rs terms, apart from those the AEC has introduced itself, because most of them occur without explanation by the scientists during the pre-AEC submission stage. Thus, it is very likely that the extent of Three Rs applications is substantially underestimated by AECs, and by those who review their activities, because of this unheralded and laudable pre-AEC activity. This paper provides some novel examples of such 'invisible' Three Rs applications to highlight this and some related points.

Keywords: three Rs applications, protocol planning, underreporting

Introduction

It is common now for animal protection laws and regulations to require that the Three Rs tenet of Russell and Burch (1959) be applied to the design of experiments involving sentient animals: i.e. *replacement* of animals with non-sentient animal or non-animal alternatives; *reduction* in the number of those sentient animals to the minimum necessary to achieve the scientific objective; and *refinement* of the procedures applied to decrease to the minimum practicable extent the negative impacts they have on the animals. The conscientious application of the Three Rs by investigators, and by animal ethics committees (AECs) when considering proposals, is an ethical requirement linked to the obligation to minimise the harm and maximise the benefits of the proposed work (Mellor and Reid, 1994; Williams et al., 2006). This, and the need to provide credible reassurance to the public that care is in fact taken to minimise the negative impacts of procedures on animals, provide impetus for Three Rs applications to be documented in approved protocols once the AEC has completed its deliberations. However, the innovative character of numerous Three Rs applications remains unrecorded because the less acceptable procedures they replace are rarely drawn to the attention of the AEC. Indeed, the AEC may have no knowledge that any Three Rs innovations have been applied apart from those the AEC has introduced itself, because most of them are included without explanation by the investigators who devised them during the pre-AEC submission stage of protocol development. Thus, it is very likely that the full extent of Three Rs applications has been and continues to be substantially underestimated by AECs, other researchers, formal AEC reviewers, regulators and concerned members of the public, because of this unheralded and laudable pre-AEC activity.

The personal experience of the present authors and discussion with numerous other animal-based scientists confirmed this state of affairs. Accordingly, the primary purpose of the present paper was to provide a preliminary report on just a few of many examples of such "invisible" or unreported Three Rs

innovations in order to alert investigators and AECs to this possibility and to stimulate thought about how they can be recorded. Our longer-term goal is to further enhance this process by providing a more extensive account encompassing a wider variety of examples drawn from all three areas of teaching, research and testing. Here we comment on one teaching and three research protocols.

Full cooperation of our colleagues with this project was ensured by prior agreement that precise details of projects, institutions and investigators would remain strictly confidential. Although this means that others cannot check specific details of each case, the examples selected serve the present purpose independently of the institutions and personnel involved. Moreover, all of the associated protocols were approved by the local AECs concerned, albeit usually without the Committees' knowledge of the full extent of the Three Rs benefits that had been implemented in each protocol.

For clarity each example is presented in the following way. A brief introduction provides background information. This is followed by details of the original protocol envisaged by the investigators. Finally, details of the modifications made to the protocol during the pre-AEC stage and the associated Three Rs benefits are outlined.

Examples

Avoiding abdominal surgery in domestic chickens – teaching protocol

Background

In order to improve students' understanding of the hormonal control of avian behaviour within the constraints of a teaching year where natural seasonal events such as puberty occur at inconvenient times, it is useful to artificially induce the behavioural changes by manipulating the hormonal balance of the birds. The purpose of this particular study was to demonstrate the impact of the pubertal onset of testosterone secretion on behaviour in domestic chickens.

Initial design

The first experimental design proposed in a draft AEC application was, before puberty and under general anaesthesia, to sham-castrate half the birds and castrate the other half, and compare their subsequent behaviours. The sham-castrated birds were expected to exhibit normal pubertal behaviour and the castrated ones no such behaviour. Unlike the majority of mammals where the testes are located in an external scrotal sac, in birds they are located in the abdomen. Accordingly, castration involves abdominal surgery with the potential for a significant negative impact on the birds during the post-surgical recovery period. Post-surgical pain can of course be mitigated by the use of analgesics, in accord with the *refinement* principle, but at the time this experiment was first proposed, analgesic protocols in birds were not well developed.

Final design

The experimental design finally submitted to the AEC proposed the use of pre-pubertal birds only: half would receive a small subcutaneous testosterone slow-release implant and the other half would receive the same implant without the testosterone in it. All implantations would be done using local anaesthetic injections, followed by a very small incision (5 mm) in the skin and a single suture. This *refinement* represented a substantial decrease in the invasiveness of the procedure and in the related potential for the birds to experience post-surgical pain and distress.

Replacing animals with *in vitro* fermentation techniques in studies of bacterial virulence factors – research protocol

Background

All animals carry bacteria. Some cause disease (they are pathogenic) and others, which are more benign (e.g. commensal or symbiotic bacteria in the gut or on the skin), do not. Some bacteria may undergo variations in ways that change their capacity to cause disease (their virulence). Studying animals infected with different bacteria increases our understanding of these bacteria, their interaction with a host and how to detect, exploit or remove them.

Initial design

The overall purpose was to introduce bacteria into naïve or previously exposed host animals via a range of routes (e.g. respiratory, gut) in order to study host-bacteria interactions and characterise the effects of disease-causing and non-disease-causing bacterial isolates. In this way the virulence characteristics of the bacteria would be determined. It was expected that disease-causing bacteria would usually produce unpleasant and distressing effects on the animals. Clearly there would be merit in avoiding or reducing these untoward effects of *in vivo* screening of microbial agents.

Final design

The original purpose remained the same, but initial steps involved *in vitro* simulation of the effect of *in vivo* conditions on the ability of the bacteria to cause disease. This was achieved by using modern fermentation equipment with fine control over key growth conditions such as nutrient availability, temperature and acidity (pH), combined with use of published bacterial genomes, the study of proteins and their functions (proteomics), advanced computer analysis of biological data (bioinformatics) and mathematical modelling. This powerful approach, made possible by advances in genetic, analytical

and computing sciences, dramatically improved the ability to define microbial virulence attributes at the molecular level and to anticipate their adverse effects on a host, thereby reducing the need for *in vivo* screening of microbial agents. This example of *replacement* of animals with non-animal alternatives avoided the need to use whole animals and to apply to the AEC for authorisation for work of this type.

Avoiding continuous and protracted indoor maintenance of deer – research protocol

Background

One determinant of the onset and waning of seasonal reproductive and related functions in farm and other animals is change in photoperiod (i.e. day-length). A common way to assess the impact of day-length in relation to other seasonal changes like temperature range, weather and feed availability, is to keep animals indoors in regulated but different light-dark cycles. The purpose of this particular study was to maintain long or short day length light conditions for deer over an extended period in order to investigate the impact on seasonal reproductive functions.

Initial design

Some deer were to be kept on a photoperiod resembling that occurring naturally and others on a reversed photoperiod. The standard approach would have been to keep the animals continuously indoors in light-controlled rooms. Although domesticated deer can be well managed for long periods indoors, it takes special skills, and, as with other animals, demands continuous practical attention to feeding, watering and mucking out. In addition, they would be confined for long periods. Accordingly, it was considered to be desirable to minimise the extent of continuous indoor housing if that were possible.

Final design

The experimental design finally submitted to the AEC allowed the deer to remain in the field for most of each day, using the normal outdoor light intensities. On a daily basis, they would be mustered in toward the end of natural daylight hours and returned to the field at predetermined times. Thus, natural daylight was combined with short periods of being held in rooms with lights on or off to achieve the required light-dark patterns. This *refinement* completely avoided any negative impact of very long lasting, *continuous* indoor maintenance of the deer, which, of necessity, would have restricted the space they had available for exercise. It also meant that the deer would be maintained on their usual outdoor diet throughout the study, and would experience all other environmental variables such as dawn, snow, wind, rain and outdoor temperature fluctuations.

Minimising animal numbers by multiple use of individual pigs – research protocol

Background

Wound healing is problematic in some human patients, particularly in the management of decubitus ulcers (pressure sores) in diabetics. A standard wound dressing is a calcium alginate membrane which is applied over the open ulcer. Regular wound dressing changes are required. Animal models are routinely used to test alternative wound dressing formulations.

Initial design

The first study design proposed in the draft AEC application involved the use of large numbers of pigs. Under general anaesthesia each animal was to be operated on to create a single pair of 12 mm diameter surgical wounds on the dorsal back region. A wound dressing, either the control calcium alginate form or the experimental test material, was to be applied over the wound area. At four specified times for up to seven days after surgery, each animal would be euthanased and the pair of wound sites excised for histological evaluation of their progress of healing. In this form, the study required the use of numerous animals to produce statistically significant results at each time point.

Final design

The study finally submitted to the AEC proposed using fewer pigs through a revised experimental design. A series of paired surgical wounds in the skin would be made on each pig over a 7-day period, with one pair made each time at 0, 3, 5, and 7 days. On each occasion the pig would be anaesthetised and wounds created with a biopsy punch. It was proposed to use a fresh biopsy punch for each wound to prevent transmission of chemical agents involved in healing (e.g. cytokines) from one wound to another. Prior wounds would be examined, measured and photographed. Test or control wound dressings would be applied to new wounds and protective bandages used to cover all sites. Any pain resulting from the wound biopsy procedure would be managed by infiltration of a long-acting local anaesthetic at the biopsy site before sampling.

At the study endpoint, therefore, each euthanased animal would provide a series of wounds at various stages of wound repair, with test and control sites in the same animal, a design facilitated by the large size of the pig. The change in study design would reduce the number of animals required to about 25% of that in the original proposal. Also, by allowing progress of wound repair to be assessed in individual animals over the whole seven-day period, it would decrease data variability and thereby allow more sensitive detection of differences between test and control dressing formulations. This case of pre-AEC re-evaluation has a predominant *reduction* emphasis, but

the additional use of long acting local anaesthetic also represents a *refinement*.

Discussion

During the collection of numerous examples of 'invisible' pre-AEC application of the Three Rs, from which the four presented here were selected, several issues became apparent. Such Three Rs applications are indeed very common and usually are not, or not often, reported to AECs. Accordingly, there would be merit in devising a means to highlight them on the application forms submitted to AECs. For instance, investigators could be required to answer the following question: *"What, if any, innovative Three Rs applications have been included during the development of this protocol?"* The purpose of this question would need to be explained, i.e. that it specifically relates to entirely new or as yet unused Three Rs applications in the context of the proposed study. This explanation would also help to ensure that the answer "none" would not be taken to imply that there had been inadequate attention to the Three Rs. Of course, a further safeguard is that there are existing questions on the forms designed to clarify what well known or routine Three Rs approaches have been included in each protocol.

For three of the present examples the answers to this question could be as follows: (1) *refinement* – small subcutaneous implants are to be inserted into domestic chickens via a 5 mm incision under local anaesthesia with one stitch, instead of general anaesthesia, abdominal surgery followed by sham or actual castration, wound closure and recovery; (2) *refinement* – deer are to be brought indoors late each afternoon to extend or restrict day-length by controlled use of lighting, leaving them free outdoors for the rest of each day, instead of having them continuously housed indoors for many months; and (3) *reduction* – instead of using different pigs for each pair of puncture wounds, the proposed multiple low-impact use of animals achieves a 4-fold reduction in the number of pigs required, and increases the fidelity and power of comparisons by reducing the potential impact of individual variation on the results. Unfortunately, with regard to the present purpose, the case of *replacement* of animals with a fermentation technique in the bacterial virulence study would not be reported to the AEC unless other aspects of the work required some use of animals. It would therefore remain unrecorded.

Collection of these examples has also highlighted the important role played by institutional animal welfare officers, designated veterinarians or animal facilities managers in stimulating thought about methodology. This usually works best when a protocol is not being developed under urgency. Leisurely and iterative reflections conducted by investigators and experienced animal welfare officers (and the like) can help investigators to think outside the conventional methodology of their discipline area. Whether working under urgency or not, it is helpful to give greater weight to the impact of the procedures on the animals rather than to the investigator's desire to achieve a particular scientific outcome, because this provides a good starting point for the fresh thinking that is required if we are to be methodologically innovative in Three Rs terms. A further benefit is the great sense of satisfaction that both investigators and animal welfare officers experience in being able to demonstrate their commitment to minimising any harm caused to the animals they use by exercising ingenuity and adopting fresh perspectives.

Finally, having now explored this form of Three Rs underreporting we have come to the view that, while useful as illustrations, the examples themselves are not the only important outcome of this enterprise. Another is the reinforcement of the concept that significant advances in the application of the Three Rs are more likely to be made when experienced professionals from allied but different disciplines work collectively to review animal-based research or teaching proposals. The previously unforeseen benefits accruing from such combined intellectual power are usually greater than those generated by an individual or a group of professionals within a restricted discipline. These examples are therefore also intended to highlight for investigators the added value of discussing deployment of Three Rs measures with experienced personnel who can provide diverse perspectives. As a pre-AEC activity this would then become even more effective and would continue to enhance, but much more 'visibly', the multi-disciplinary perspectives that AEC members themselves provide.

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