Special issue

Animals and the 3Rs in toxicology research and testing: The way forward

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Abstract
Despite efforts to eliminate the use of animals in testing and the availability of many accepted alternative methods, animals are still widely used for toxicological research and testing. While research using in vitro and computational models has dramatically increased in recent years, such efforts have not yet measurably impacted animal use for regulatory testing and are not likely to do so for many years or even decades. Until regulatory authorities have accepted test methods that can totally replace animals and these are fully implemented, large numbers of animals will continue to be used and many will continue to experience significant pain and distress. In order to positively impact the welfare of these animals, accepted alternatives must be implemented, and efforts must be directed at eliminating pain and distress and reducing animal numbers. Animal pain and distress can be reduced by earlier predictive humane endpoints, pain-relieving medications, and supportive clinical care, while sequential testing and routine use of integrated testing and decision strategies can reduce animal numbers. Applying advances in science and technology to the development of scientifically sound alternative testing models and strategies can improve animal welfare and further reduce and replace animal use.

Keywords
Animal welfare, alternative methods, humane endpoints, refinement, reduction, replacement

Introduction
Despite widespread efforts to eliminate the use of animals in testing over the past 35 years, animals are still widely used for safety assessments and efficacy testing of nearly all new chemicals, pesticides, consumer products, drugs, medical devices, vaccines, and many other products. Such animal research and testing is necessary to address regulatory requirements and to protect and advance the health of people, animals, and the environment. While regulatory authorities have approved at least 63 alternative methods, safety testing still uses large numbers of animals and involves significant pain and distress. Research using in vitro and computational models has dramatically increased in the last decade; however, such efforts have not yet measurably impacted animal use for regulatory testing and are not likely to do so for many years or even decades. Before new tests can replace animals, they must be shown to provide equivalent or improved protection as the animal test that they are proposed to replace. The test must then be accepted by regulatory authorities and implemented by regulated industry in order to actually avoid the use of animals. This article will discuss opportunities and approaches urgently needed to eliminate pain and distress and to reduce animal numbers in testing, while research, development, and validation necessary to achieve full replacement of animals are pursued.

The 3Rs
Public concerns about the welfare of animals in research and testing have led to laws and policies that aim to reduce or avoid animal pain and distress, ensure that animals are only used when scientifically justified, and ensure that only the fewest animals necessary for scientifically valid results are used. This approach to the humane use of animals is commonly referred to as the “3Rs of Alternatives,” a concept
first described in 1959 by William Russell and Rex Burch in their book *The Principles of Humane Experimental Technique*. The concept involves refining animal use to lessen or avoid pain and distress and enhance animal well-being, reducing the total number of animals required for specific studies, and replacing animals with nonanimal systems and approaches.

**Regulations and the 3Rs**

Nearly all countries now have laws, policies, and regulations that require consideration of 3Rs alternatives and review by oversight committees before animals can be used in research or testing. In the United States, the Animal Welfare Act (AWA) requires compliance with AWA regulations for warm-blooded animals used in research and testing. AWA regulations require consideration of alternatives whenever procedures involve more than slight or momentary pain or distress. Institutional Animal Care and Use Committees (IACUCs) are required to review this consideration of alternatives before approving the use of animals in potentially painful or distressful procedures. IACUCs must also approve any potentially painful procedures that will be conducted without pain-relieving drugs, which include many types of toxicity studies. Proposals to use animals must also provide a rationale for involving animals and for the appropriateness of the species and numbers of animals to be used.

However, many animals used in testing are not covered by AWA regulations. For example, laboratory rats (*Rattus* spp.), laboratory mice (*Mus* spp.), and birds bred for use in research are specifically not covered by AWA regulations, despite the fact that rats and mice account for the vast majority of the animals used in research and testing. Unless specifically covered by the Public Health Service (PHS) Policy on the Humane Care and Use of Laboratory Animals, such as research funded by the PHS, there is no requirement for IACUC review or consideration of 3Rs alternatives when testing procedures involve pain and distress for noncovered species. Full consideration of available alternative test methods and IACUC oversight of noncovered animal species used in testing would contribute to improving and ensuring their welfare.

**Animal pain and distress in testing**

Animals that develop toxic injury or disease during toxicology research and testing studies often experience significant pain, distress, suffering, and/or death. Nearly all of the estimated 2000 new chemicals and thousands of chemical products introduced each year must undergo acute safety tests to determine whether the substance is toxic or lethal and whether it causes allergic skin reactions or damage to eyes or skin. Much of this acute testing is still conducted using mice, rats, guinea pigs, and rabbits, with many animals experiencing death, acute illness, pain, and distress from toxic effects. Many substances also require chronic toxicity and carcinogenicity studies that use over 600 rats and mice dosed for 2 years, during which up to 50% of the animals die or must be euthanized before the end of the study because of severe chronic disease and/or excessive tumor burden.

Each facility using animals for research and testing must file an annual report with the United States Department of Agriculture (USDA) that lists the number of animals that experience more than slight or momentary pain or distress without pain-relieving medications. An explanation of the procedures producing the pain or distress and the reasons that drugs were not used must also be provided. The 2013 USDA Annual Report of Animal Usage documented 85,325 animals that experienced unrelieved pain and distress in fiscal year 2013. However, it is important to note that the USDA reports do not include rats and mice, which are estimated to account for 95% of the animals used annually in research and testing. A detailed review of another USDA report (2010) found that testing procedures accounted for 94.7% of the animals that experienced unrelieved pain or distress. Of these, 40% were used for safety and drug efficacy testing, while 60% were used for vaccine testing.

**Federal agency 3Rs mandates**

The United States and many other countries have established government centers and organizations charged with developing, validating, and gaining regulatory acceptance of 3Rs alternative methods. In the United States, the National Institutes of Health is charged with supporting 3Rs research, and the National Institute of Environmental Health Sciences (NIEHS) is charged with developing and validating alternative methods for acute and chronic toxicity testing. The US Interagency Coordinating Committee on the Validation of Alternative Methods
(ICCVAM) is charged with reducing, refining, and replacing the use of animals in testing, evaluating alternative test methods, and forwarding formal recommendations to federal agencies for acceptance decisions. As a result of efforts by NIEHS, ICCVAM, and centers in other countries, 63 alternative test methods have now been accepted by regulatory authorities, including 28 animal-based methods that refine or reduce animal use and 35 methods that do not use animals.1,2

**Implementation of alternative methods**

While regulatory authorities have accepted numerous alternative test methods, many of these test methods have not been widely implemented. In order to fully impact animal welfare, regulatory agencies and industry must implement and use accepted alternative methods. There are several barriers to implementing accepted alternative methods that must be overcome. First, industry must be assured that government agencies will accept data from accepted alternative methods. US agencies must therefore signal their willingness to accept data from scientifically valid alternative methods by updating and incorporating accepted alternative methods in their regulations, guidelines, and guidances, as appropriate.4 For example, federal regulatory agencies have indicated their acceptance of all alternative test methods recommended by ICCVAM.13 However, few of these test methods have actually been incorporated into agency guidelines and/or guidances. Such incorporation is a crucial step to actual use of the methods by industry, which uses federal agency test guidelines and guidances to develop test protocols compliant with good laboratory practices.

As an example, all US federal agencies have accepted several alternative test method recommendations for eye irritation testing. These include acceptance of in vitro methods in 2006 and 2010, and the routine use of pain-relieving medications and additional humane endpoints in 2010.14-16 However, the 1998 Environmental Protection Agency health effects test guideline for eye irritation testing remains unchanged.17 If regulatory testing guidelines are not updated to include or reference accepted alternative methods, studies may continue to be conducted without the benefit of pain-relieving agents, humane endpoints, or in vitro methods. Timely updating of regulatory guidelines is essential to ensure the consideration and use of in vitro methods and procedures to reduce or avoid animal suffering.

A second impediment to implementation relates to the use of internationally accepted alternative test methods. The United States and 34 other countries are members of the Organization for Economic Cooperation and Development (OECD) and have agreed to accept data from tests conducted in accordance with OECD test guidelines under a Mutual Agreement of Data Treaty.1 However, reluctance persists by some companies to use these internationally accepted test methods if there is not a clear indication of acceptance by the respective regulatory authorities in each country where the product will be marketed. Therefore, federal agencies should promote and actively signal their willingness to accept results from OECD-adopted test guidelines for alternative methods in order to ensure their consideration and use where appropriate.

A third impediment to implementation is that reviewers and other staff in regulatory agencies are not always aware of or familiar with accepted alternative methods. As a result, they may not discuss or suggest the consideration of potentially relevant alternative methods to the regulated community. The timely education and training of regulatory staff about the usefulness and limitations of available alternative methods is clearly important for effective implementation.

**Opportunities for reducing pain and distress**

With large numbers of animals experiencing unrelied pain and distress in testing, there is an urgent need to address and eliminate the sources of pain and distress. Numerous opportunities and approaches are available that could contribute to eliminating the current suffering experienced by animals in testing.5,6,18–20 Such refinements not only could provide for improved animal welfare but could also enhance the quality of experiments by reducing or eliminating pain and distress as an experimental variable. Strategies to reduce or avoid pain and distress include (1) using earlier more humane endpoints, (2) using pain-relieving medications, and (3) providing appropriate veterinary and supportive clinical care.

Humane endpoints are criteria that can serve as the basis for ending a test procedure earlier in order to terminate or avoid pain and distress while still attaining study objectives.5,6,20,21 Using earlier humane endpoints can reduce the severity and/or duration of pain and distress experienced by an animal.5,6,20,21 Clinical signs, physiologic and biochemical measurements,
and other parameters can serve as potential earlier biomarkers of humane endpoints. Humane endpoints are especially useful when medications cannot be used to treat pain and distress due to interference with study objectives. The ideal humane endpoints are those that can be used as criteria to end a procedure before the onset of animal pain and distress, while still attaining the scientific or testing objective.\cite{5,6,20,21}

The murine local lymph node assay (LLNA) is an example of an alternative test method that incorporates an earlier mechanistic humane endpoint that completely avoids pain and distress previously involved in testing to determine the allergic contact dermatitis (ACD) potential of chemicals.\cite{1,5,6,18,20} The traditional test for ACD, the guinea pig maximization test (GPMT), requires the actual elicitation of allergic dermatitis, which is manifested by redness, swelling, and pruritus. The GPMT also involves the intradermal injection of complete Freund’s adjuvant, which results in significant pain and distress from severe skin irritation, inflammation, and necrosis. In contrast, the LLNA measures an adverse outcome pathway event that occurs during the induction phase of ACD and that is used as the predictive humane endpoint for the study, thus avoiding the need to progress to the elicitation phase which involves a painful endpoint.\cite{5,6,18} Despite the acceptance of the LLNA as an approved alternative for ACD testing in 1999, review of the justifications for unrelied pain and distress for the 2010 USDA report found 4454 guinea pigs in this category that were used for ACD testing.

Anesthetics and analgesics are a second strategy that can be used to reduce or avoid pain and distress in certain testing situations.\cite{5,6} For example, the OECD updated its ocular test guideline in 2012 to incorporate routine pain management procedures that should always be used in animal studies to determine the eye injury potential of test substances.\cite{5,16,22} This guideline incorporates the routine use of topical anesthetics and systemic analgesics prior to the application of test articles to the eye. Systemic analgesics are then continued until eye lesions resolve or the study is terminated. Frequent evaluation and recording of eye injuries is also conducted throughout the study, including observation for clinical signs and lesions that can serve as humane endpoints to terminate the study early. Pain-relieving medications should be considered and evaluated for their potential usefulness in reducing or eliminating pain and distress for other types of testing that still involve painful procedures or toxicity.

The third strategy for reducing or avoiding pain and distress and suffering is the use of appropriate veterinary and clinical supportive care.\cite{5,6} Such care is essential for assuring animal well-being and should always be provided to minimize discomfort in animals used in research and testing studies. This care includes frequent observation of animals to identify clinical signs and the provision of care necessary to reduce or alleviate pain and distress and to address injuries or other adverse effects. Appropriate interventions should be made to ensure animals maintain good hydration, can readily access water and food, and have a clean and dry environment. Serious consideration should also be given to providing supportive clinical care during preclinical drug and device efficacy testing as would be provided to a human test subject with the same disease or injury.\cite{23} Such care would allow for the animal subjects to more accurately model the actual clinical progression in humans. Similarly, safety testing in animals conducted under clinical conditions corresponding to those of human patient counterparts would allow for identification of toxic effects relevant to the product without the confounding effect of nonspecific causes. Such clinical care would include appropriate treatment of pain and distress, as would be done in the human clinical setting, thereby avoiding or reducing pain as a confounding variable.\cite{23}

**Opportunities for animal reduction**

While many regulatory tests still require the use of large numbers of animals, the number of animals used for each study could potentially be reduced while still allowing attainment of the testing objective. Sequential testing and integrated testing and decision strategies (ITDS)\cite{1,3} are two approaches that can potentially reduce the number of animals in a study. These approaches should routinely be considered during the design of individual toxicology studies and should also be evaluated for their potential for incorporation into standardized regulatory testing guidelines in order to further reduce animal use.

Sequential animal testing has been successfully used in conjunction with an innovative statistical approach to significantly reduce animal use for acute oral toxicity testing. The test, referred to as the up-and-down procedure (UDP), typically uses only uses three to seven animals per test, which is an 80%
reduction compared with the traditional test.\textsuperscript{5,6,24,25} The successful application of sequential testing in the UDP suggests that its consideration and application to other testing protocols may have the potential to significantly reduce animal use for testing.

Sequential testing has also been incorporated into OECD test guidelines for ocular and dermal irritation testing.\textsuperscript{22,26} In these testing protocols, the test is considered complete if the first animal develops severe lesions or if the first and second animal develop concordant negative or mild-to-moderate responses. While such testing takes more time to conduct than dosing all animals at the same time, it can reduce the total animals required as well as the number of animals that experience pain and distress.

ITDS can reduce animal use by considering and using multiple sources of data and information in a stepwise manner before using animals.\textsuperscript{1,3,18,27} At each decision stage, all of the available information is considered to determine if a hazard classification decision can be made, or if not, what additional information has the greatest likelihood of informing a decision. Using ITDS can also increase the certainty of hazard and safety predictions beyond that associated with only a single source of data or information derived from a specific testing study.

As an example, ITDS can be applied to acute oral toxicity to further reduce animal use. All available existing information is first considered, including any human accidental exposure data. If there is not sufficient information to make a hazard decision, then additional data would be generated, which might include using an in vitro cytotoxicity test to estimate the relative toxicity of the test article.\textsuperscript{28} If this information is not sufficient for a decision, then the in vitro data may be used to help establish an initial starting dose for the animal study. Such an approach has been shown to further reduce the number of animals required for acute oral toxicity testing.\textsuperscript{28,29} ITDS approaches are also being used to reduce the number of animals for skin sensitization testing.\textsuperscript{18}

**Opportunities for animal replacement**

The replacement of animal models for toxicological testing will ultimately provide the greatest impact on animal welfare by completely avoiding the need to use animals. For example, full replacement now appears to be feasible for estimating local toxicities. Some other notable replacement models include the replacement of most rabbits for pyrogen testing by the bacterial endotoxin and human monocyte activation tests,\textsuperscript{5,30} and an engineered cell line approved as a substitute for the mouse potency test for lot release of botulinum therapeutic products.\textsuperscript{5,31} However, complete replacement of animals to assess systemic toxicities will require extensive understanding of all the cellular and molecular events and pathways that can lead to adverse outcomes.\textsuperscript{32–34} Additionally, uncertainties related to absorption, distribution, metabolism, and excretion must be overcome. Other challenges include eliminating extrapolation uncertainties for factors such as age, gender, ethnicity, genetic susceptibilities, and co-morbidities in target populations.

**The way forward**

Animal welfare in toxicology research and testing has benefited from the regulatory acceptance of numerous alternative methods that reduce, refine, and replace animal use for selected studies. However, the complete replacement of animals in toxicology research and testing remains a complex and challenging task that will take many years to accomplish. In the meantime, there are excellent opportunities to advance the welfare of animals by eliminating pain and distress, and systematically reducing the number of animals necessary for individual studies. Future progress will depend on the level of support provided to develop new refinement and reduction strategies and to carry out the technical, biological, and regulatory validation studies necessary to gain regulatory acceptance of 3Rs alternative test methods. Efficient translation of toxicity pathways and biomarkers into earlier more humane endpoints will contribute to reduced pain and distress for animals still required for regulatory testing. Reduction of animal use will be made possible by incremental application and incorporation of mechanistic knowledge gained from in vitro and computational models into ITDS. Timely regulatory acceptance of methods and strategies found to be scientifically valid, and active promotion of their implementation by reviewers and regulated industries will contribute significantly to improved animal welfare in testing. The continued discovery and application of new science and technology to develop and validate scientifically sound models and earlier humane endpoints can be expected to improve animal welfare and further reduce and replace animal use, while ensuring and
advancing the health of people, animals, and the environment.

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