

Humane Endpoints for Laboratory Animals Used in Regulatory Testing

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Abstract

Laboratory animals are used for regulatory testing to assess the safety, efficacy, and/or potential adverse health effects of new chemicals and products such as vaccines, medicines, food additives, pesticides, and industrial chemicals. Testing results are used for risk assessment decisions intended to safeguard human and animal health. However, chemical toxicity and vaccine testing can cause injury, disease, and mortality involving significant pain and distress. Alleviation of pain and distress in animals during testing is problematic because regulations allow treatment only if the treatment does not interfere with the study. One approach to this problem has been to identify criteria that can serve as the basis for ending a test procedure sooner in an effort to terminate or avoid pain and distress while still allowing attainment of study objectives. These criteria are referred to as humane endpoints because they reduce the severity and/or duration of pain and distress experienced by an animal. New and revised test methods and approaches that incorporate humane endpoints are being considered and adopted by national and international regulatory testing authorities. The prerequisite for adoption of these methods is a determination that the methods have been adequately validated and that they provide equivalent or better information for risk assessment. Further progress in reducing animal pain and distress resulting from regulatory testing is expected as scientific and technological advances are incorporated into testing procedures and strategies.

Key Words: alternatives; humane endpoints; laboratory animals; pain and distress; refinement; regulatory testing; toxicology

Introduction

Regulatory testing requires the use of animals to assess the safety, efficacy, and/or potential adverse health effects of new chemicals and products such as vaccines, medicines, food additives, pesticides, and industrial chemicals. This testing information provides the basis for

risk assessment decisions to safeguard human and animal health. However, adverse effects on animals can be caused during such testing from acute and chronic chemical toxicity and by induced infections that occur during vaccine potency testing. Resulting injury, disease, and mortality can involve significant pain and/or distress in experimental animals.

Pain management for laboratory animals was addressed in original animal protection laws and is addressed in current animal care and use policies (PHS 1996), guidelines (NRC 1992), and regulations (USDA 1998a). More recently, clinical pain management standards have also been established for humans, with recognition that pain can be a common part of the patient experience and that unrelieved pain has adverse physical and psychological effects (JCAHO 1999). Clinical standards assert that all patients have a right to pain relief (JCAHO 1999). The goal of these standards is to ensure that all pain in humans is appropriately managed. Both human and animal pain management guidelines and standards seek to minimize the occurrence, duration, and severity of unrelieved pain.

As stated in animal welfare regulations, procedures that cause more than momentary or slight pain and distress should be performed with appropriate sedation, analgesia, or anesthesia (USDA 1998a). However, some research and testing studies involve pain that cannot be relieved with drugs because they would interfere with the scientific objectives of the study. To avoid possible confounding effects from pain-relieving medications, animals used in testing are rarely treated with such drugs. Testing regulations allow treatment of animals only if the treatment does not interfere with the study (EPA 1998; FDA 1999).

When animals must experience unrelieved pain and distress, federal regulations and policies mandate that discomfort must be limited to what is unavoidable for the conduct of scientifically valuable research and that the pain and distress should continue only for the duration necessary to accomplish the scientific objectives (USDA 1998a). As further stated in the Public Health Service Policy on the Humane Care and Use of Laboratory Animals, animals that would otherwise suffer unrelieved severe or chronic distress should be painlessly killed at the end of the procedure or, if appropriate, during the procedure (PHS 1996).

The number of animals experiencing unrelieved pain and distress in the United States cannot be determined accurately because there are no reporting requirements for rats and mice, two of the most commonly used species in tox-

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icity testing. However, unrelieved pain and distress must be reported for animal species regulated by the US Department of Agriculture, and these data can be used to estimate the total number of animals in this category. For example, in 2000, unrelieved pain and distress was reported for 104,202 animals, or 7.4% of all reported animals (USDA 2001). Because an estimated 90% of animals used in research and testing are rats and mice, the total number of laboratory animals experiencing unrelieved pain and distress annually may exceed one million. Data from both the United States and Canada indicate that regulatory testing accounts for the majority of the animals experiencing unrelieved pain and distress (CCAC 1998; Stephens et al. 1998).

Is it possible to reduce unrelieved pain and distress in laboratory animals used in testing? As described in several recent reports, it appears that new test methods and approaches can further reduce animal pain and distress (Hendriksen and Morton 1999; ICCVAM 1999a,b; Stokes 2000b). The approach involves the use of criteria that can serve as the basis for ending a test procedure sooner to terminate or avoid pain and distress while still attaining study objectives. These criteria are referred to as *humane endpoints* because they reduce the severity and/or duration of pain and distress experienced by an animal (Stokes 2000a,b). Ultimately, criteria that can be used to end studies *before* the onset of pain and distress are the ideal humane endpoints. This presentation is a discussion of current humane endpoints for animals used in regulatory testing and a discussion of a process for establishing humane endpoints.¹

Current Humane Endpoints for Safety Testing

Toxicity testing in animals typically involves exposure to a test article and observation of animals for signs of toxicity for a specific period of time. If toxicity is produced before the end of the scheduled study, it may result in animals experiencing pain and distress as a result of localized tissue damage such as eye or skin irritation or systemic toxicity involving damage to various organs and tissues. Pain and distress may also result from the development of neoplasia and chronic disease and from development of infections in unprotected animals during vaccine testing. As stated in current regulatory testing guidelines, animals obviously in pain, showing signs of severe and enduring distress, or characterized as moribund should be humanely killed rather than allowed to survive to the end of the scheduled study (EPA 1999; OECD 2000). Thus, clinical signs of severe pain and distress and moribund condition currently serve as endpoints that can reduce the duration of pain and distress.

Moribund Condition as a Humane Endpoint

Moribund condition, defined as “in the state of dying” or “at the point of death” (Toth 1997, 2000), is a commonly used endpoint in testing. Pre-emptive humane killing of moribund animals can prevent further pain and distress that might occur before spontaneous death and thus serves as a humane endpoint. However, humane killing of moribund animals does not eliminate the pain and distress that an animal may experience during progression to a moribund condition.

Although death is not a required endpoint for routine toxicity testing and most vaccine potency testing, animals are often found dead during studies. Establishing procedures to detect and humanely kill moribund animals can reduce the number of animals that die spontaneously. Further reductions in spontaneous deaths can be achieved by developing and using objective criteria that are predictive of impending death and that can be used as the basis for timely euthanasia (Toth 2000). In addition to reducing animal pain and distress, moribund euthanasia allows for the collection of tissues and other biological specimens that may otherwise be lost or rendered unusable when an animal is found dead.

Various clinical signs are indicative of a moribund condition in laboratory animals (Tomasovic et al. 1988; Toth 1997). These signs typically include one or more of the following:

- impaired ambulation, which prevents animals from reaching food or water;
- excessive weight loss and extreme emaciation;
- lack of physical or mental alertness;
- difficult labored breathing;
- prolonged inability to remain upright.

It is important to observe animals frequently enough to detect signs of impending death so that animals can be humanely killed in a timely manner. A minimum of twice daily observation is recommended, with more frequent observations immediately after dosing and when increased morbidity or mortality is expected. Animals not likely to survive until the next scheduled observation should normally be humanely killed. For testing procedures in which animals are often found dead, more frequent and careful observation for moribund animals should be considered to reduce spontaneous deaths.

Humane killing of animals that are moribund or experiencing severe pain or distress should always be done in a manner that produces the least possible amount of additional pain or distress. Guidance is available for methods that are considered humane and that are generally acceptable for animals used in toxicology studies (AVMA 2000; EC 1997). Methods commonly used in toxicology studies include carbon dioxide-oxygen mixtures for rodents and injectable euthanasia preparations for nonrodent species.

¹This manuscript does not reflect official government agency policy.

Establishing Clearly Defined Humane Endpoints

Proposed protocols for testing studies should always have clearly defined endpoints describing when animals can be removed from a study for humane reasons. The potential for development of pain and distress from proposed procedures should also be described in protocols. If pain or distress is anticipated, a detailed plan should be provided for when and how it will be alleviated. This plan should include detailed written criteria for the endpoints that will be used to determine when animals can be removed, treated, or humanely killed. Procedures should be based on clear statements regarding who can make the decision to kill animals and how to proceed if a situation arises on weekends, holidays, or in the absence of the responsible study director. Even if pain and distress are not anticipated, every protocol should contain a contingency plan for dealing with potentially unexpected situations.

Recognition and Assessment of Pain and Distress

Guidance on recognizing and assessing pain and distress in laboratory animals has been reviewed by others (CCAC 1998; Montgomery 1990; Morton 1990, 2000; NRC 1992; OECD 2000; Olfert 1996). Guidelines for choosing appropriate endpoints in experiments using animals for research, testing, and teaching have also been developed (CCAC 1998; OECD 2000). Procedures that cause pain or distress in humans should also be considered to cause pain and distress in animals unless the contrary is established (PHS 1996). Due to the inability of animals to verbalize, it is important for animal care staff and researchers to know how to recognize clinical signs of pain and distress (Carstens and Moberg 2000).

General IACUC Considerations for Humane Endpoints

Institutional animal care and use committees should ensure that accepted best practices for testing procedures involving potential pain and distress are considered and incorporated into animal study protocols. A sound scientific rationale should be provided for any deviations from established best practices. In the United States, if more than momentary or slight pain and distress will not be relieved with appropriate analgesics or anesthetics, the principal investigator must provide a written explanation of the scientific rationale for withholding such agents (USDA 1998a). The principal investigator must also provide an assurance that unrelieved pain will continue for only the minimum period of time necessary to attain the study objectives (PHS 1996; USDA 1998a). The institutional animal care and use committee must determine that the discomfort and pain to animals will

be limited to an amount that is unavoidable for the conduct of scientifically valuable research or testing (PHS 1996).

Humane Endpoints for Acute and Chronic Toxicity Testing

Test methods for acute lethality, dermal and ocular irritation/corrosion, and chronic toxicity/carcinogenicity are among those that may involve significant pain and distress or spontaneous deaths. Over the past several years, new testing guidelines, procedures, and models have been proposed and adopted that directly or indirectly involve more humane endpoints. These practices can reduce pain and distress compared with traditional testing procedures.

Acute Lethality Testing

Acute lethality testing is performed to estimate the dose expected to kill half of the animals (i.e., median lethal dose or LD₅₀) of a substance after oral, dermal, or inhalation administration of a single dose. Standard acute oral toxicity testing involves administering a test article to three dose groups of rats and observing the animals for lethality for 14 days (EPA 1998; OECD 1987). However, the use of newer procedures, humane endpoints, and in vitro methods can potentially reduce the severity and duration of pain and distress for acute lethality testing (Sass 2000; Schleder et al. 2000).

For example, current testing guidelines state that moribund animals may be humanely killed, and these events are to be considered the same as natural deaths (EPA 1998; OECD 1987). International guidelines for using clinical signs as humane endpoints for animals in safety evaluations are available that can be helpful in avoiding spontaneous deaths (OECD 2000). The fixed dose procedure for acute oral toxicity calls for animals to be humanely killed when signs of evident toxicity are observed (OECD 2001a). In this procedure, clinical signs of evident toxicity serve as humane endpoints that precede death or moribund condition. Two other test methods for acute oral toxicity, the acute toxic class method (OECD 2001b) and the up-and-down procedure (ICCVAM 2001c; OECD 2001c), use traditional endpoints but require fewer animals than the conventional test procedure.

In vitro cytotoxicity methods may be useful in reducing the number of animals used and the number of deaths that result from toxic substances in acute oral toxicity testing (ICCVAM 2001a,b; Spielmann et al. 1999). For some substances, data from in vitro cytotoxicity tests can help estimate the in vivo median lethal dose, which can then be used as the basis for a starting dose for in vivo testing. This approach can reduce both the number of animals used in a procedure and the number of deaths that occur when a test substance is toxic.

Skin Corrosivity Testing

The rabbit has traditionally been used to evaluate whether substances are corrosive to skin (EPA 1999). However, four in vitro methods have been accepted by regulatory authorities that can be used to identify most corrosive substances: Corrositex®, Episkin™, Epiderm™, and the rat skin transcutaneous electrical resistance method (DOT 2000; Fentem et al. 1998; ICCVAM 1999a; Liebsch et al. 2000; NIEHS 2001b; Stitzel 2002). If the in vitro test result is positive, no further animal testing for corrosivity is usually necessary. If the in vitro test is negative, observations in an in vivo dermal irritation test can be used to confirm that it is noncorrosive. Only a small percentage of animals would be expected to develop corrosive lesions in the confirmatory test. Sequential testing is also recommended so that if a corrosive lesion is produced in the first animal, no further testing is required (OECD 2001a). Furthermore, current guidelines include the recommendation that when a corrosive effect is observed, the test can be immediately terminated and the animal humanely killed (OECD 2001d). The use of in vitro methods and this earlier in vivo endpoint will thus eliminate or minimize, respectively, pain and distress for corrosivity testing.

Ocular Irritation Studies

An ocular irritation tiered testing strategy has recently been adopted that incorporates earlier endpoints to minimize pain and distress and also minimizes animal use (OECD 2001d; Sass 2000; Stitzel 2002). The first tier of the strategy involves a weight-of-evidence decision based on a review of available toxicity data for related substances. Sufficient information that is available to support classification as highly irritating or corrosive can obviate the need for animal testing. Other situations in which classification might be made without further testing include the following:

- pH value <2 or >11.5 (e.g., strong acids or bases);
- a known severe dermal irritant or corrosive;
- dermal toxicity (lethality) <200 mg/kg.

If a substance cannot be conclusively predicted as highly irritating or corrosive, it is tested in one animal. If severe ocular lesions are produced, it is classified without further animal testing (OECD 2001d; Springer et al. 1993).

If a severe corneal opacity score is present 24, 48, or 72 hr after administration of the test substance, such lesions are usually irreversible and those animals should normally be humanely killed (Gupta et al. 1993). Local or systemic analgesics should be considered if there is potential for a test substance to cause pain or if initial testing indicates a painful reaction (Gupta et al. 1993; Stitzel, 2002). However, one must be cognizant of potential alterations of ocular response when using analgesic agents (Durham et al. 1992).

Chronic Rodent Toxicity and Carcinogenicity Studies

Rodents used in long-term studies may develop a range of chronic and spontaneous diseases manifested by observable clinical signs and conditions (Kuijpers and Walvoort 1991; Maronpot et al. 1999; Montgomery 1990). A number of clinical signs may indicate irreversible conditions that involve or may progress to pain and distress. These clinical signs and other measurable parameters can therefore serve as earlier humane endpoints. For example, significant body weight loss may indicate severe irreversible liver, kidney, or other organ impairment, or secondary effects from neoplasia. Increasing the weighing frequency to weekly after 12 mo until the end of the study has been recommended to improve detection of animals with serious diseases or extensive cancers (Kuijpers and Walvoort 1991). Clinical chemistry may also be helpful in identifying critical target organ failure and impending death, allowing for animals to be humanely killed.

Increasing the frequency and level of monitoring after the onset of tumor development can allow for appropriate intervention before significant deterioration or death (Wallace 2000). Effective monitoring systems and endpoints should include limits on the tumor burden and severity of tumor-associated disease. Altered physiological, biochemical, and other biomarkers have been suggested as endpoints that are potentially more objective and reproducible than clinical signs (Wallace 2000).

Genetically engineered animal models are being used increasingly to evaluate the carcinogenicity of substances (Contrera and DeGeorge 1998). As experience accrues with each specific genotype, phenotypic alterations that adversely affect animal well-being can be identified and anticipated. Animal study protocols should include plans for addressing both expected and unexpected adverse outcomes for genetically altered animal models (Dennis 2000), especially during development and validation stages.

Humane Endpoints for Vaccine Potency Testing

New batches of vaccines must be tested to ensure that they are safe and can provide protective immunity. This routine testing typically involves immunization of several groups of animals with different dilutions of the vaccine, followed by exposure of the animals to the infectious agent of interest. Animals with insufficient protective immunity develop induced infections. Unprotected animals often develop disease which is frequently lethal. Although regulatory authorities have in the past typically required death as an endpoint for such studies, some authorities now allow humane killing of moribund animals (USDA 1998b).

Sensitive biomarkers of early infection can often be identified for infectious agents used in protective immunity studies. For example, earlier humane endpoints for vaccine

potency testing have now been developed and validated for potency testing of *Pertussis* and rabies vaccines (Hendriksen and Steen 2000). Quantitative physiological and biochemical changes during infection have also been found to be early predictors of death (Olfert and Godson 2000). For example, specific decreases in body temperature are effective early predictors of eventual death for some infections in rodents, and increased levels of acute phase proteins resulting from cytokine production during infections have been found to be useful earlier endpoints (Olfert and Godson 2000).

Hind limb paralysis and a decrease in body temperature to less than 34.5°C, which are predictive of impending death for *Pertussis*-infected animals, have been proposed as humane endpoints for *Pertussis* vaccine testing (Calver et al. 2000; Cussler et al. 2000). Specific weight loss and the presence of specific neurological signs, which are predictive of eventual death in unprotected rabies-infected mice, have been proposed as humane endpoints for rabies vaccine testing (Cussler et al. 2000).

Development, Validation, and Implementation of Humane Endpoints

Current humane endpoints for testing typically use overt clinical signs that are highly predictive of a certain adverse effect or death. However, progression to these clinical conditions typically involves pain and distress. Thus, further reduction or elimination of pain and distress for many types of testing will be accomplished only by developing earlier endpoints that are more humane. One approach is to identify one or more critical pathophysiological events leading to the adverse outcome of interest and developing these as earlier mechanistic endpoints. Before they are used in lieu of traditional endpoints, both clinical and mechanistic endpoints must be evaluated to determine their validity. A systematic approach for developing and validating humane endpoints is summarized in Figure 1 (Stokes 2000a). The first stage is to identify test methods that still use endpoints wherein animals may experience pain and distress.

Research and Development

The second stage involves identification of the initial mechanism of action event or one or more subsequent critical mode of action events leading to toxic injury or disease (Figure 1). This step may require additional laboratory research. The third stage is the identification and development of detectable biomarkers linked to the mechanism or mode of action events that can serve as earlier experimental endpoints. These biomarkers may be clinical, pathological, physiological, or behavioral alterations. Biomarkers may be readily observable (e.g., distinct clinical signs) or measurable (e.g., body temperature or blood pressure changes). They may also consist of detectable alterations in tissues or

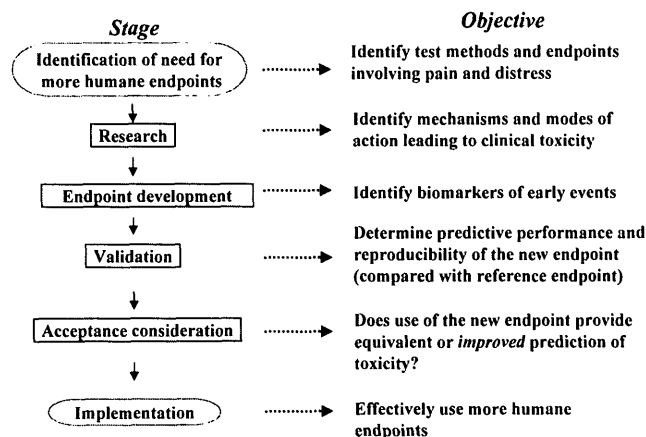


Figure 1 Process for developing humane endpoints for toxicity testing. Reprinted with permission from Stokes WS. 2000. Humane endpoints for laboratory animals used in toxicity testing. In: Balls M, van Zeller AM, Halder M, eds. Progress in the Reduction, Refinement, and Replacement of Animal Experimentation. Amsterdam: Elsevier. p 897-906.

body fluids (e.g., certain serum chemistry parameters). Specific biomarker values should be established that are predictive of the toxic effect of interest and can be used as the basis for removing animals from the study.

Validation

The fourth stage requires validation of the biomarker as an earlier endpoint. This step involves assessing the performance of the new endpoint for predicting the adverse effect of interest compared with the existing endpoint. The intra- and interlaboratory reproducibility of the new endpoint should also be determined. Validation studies should be designed to ensure that established validation criteria are adequately addressed (Balls and Karcher 1995; ICCVAM 1997; OECD 1996). In the United States, the Interagency Coordinating Committee on the Validation of Alternative Methods provides a process by which new testing methods that incorporate more humane endpoints can be evaluated and proposed for regulatory adoption (ICCVAM 2000; Stokes 2000a; Stokes and Hill 2000).

Acceptability

The fifth stage requires determination of the acceptability of the new endpoint. This step involves evaluation of the validation study results and all other relevant information on the new approach or method. The new endpoint should provide an equivalent or improved prediction of the adverse effect of interest compared with the current endpoint (ICCVAM 1997; OECD 1996). The new method should also meet

other established acceptance criteria for new and revised test methods (ICCVAM 1997; OECD 1996).

Implementation

If the new endpoint is accepted by regulatory authorities, then regulated communities and others should be informed and the new endpoint implemented as preferred practice. Data substantiating the validity of the new endpoint should be published in scientific journals. Finally, training should be provided for individuals responsible for directing, conducting, or reviewing the new test method.

Murine Local Lymph Node Assay: Example of a Mechanistic Humane Endpoint

The murine local lymph node assay (LLNA²) is an example of a test procedure that eliminates pain and distress by incorporating an earlier mechanistic endpoint (ICCVAM 1999b; Kimber et al. 1998). The LLNA is based on research indicating that lymphocyte proliferation is an essential event during the induction phase of allergic contact dermatitis and can serve as an early biomarker of allergic contact dermatitis. In the LLNA, lymphocyte proliferation is measured in the draining lymph nodes of animals topically exposed to the test substance. Because only the induction phase of sensitization must be assessed, there is no need to evaluate the elicitation phase in which sensitizing substances produce erythema, swelling, and pruritus. Other advantages of the LLNA are that it uses fewer animals, involves a much shorter time to perform, and does not require the use of potentially irritating adjuvants commonly used in the traditional guinea pig test (ICCVAM 1999b; Stokes 2000a). The LLNA was recently recommended as a valid alternative test method for assessing the allergic contact dermatitis potential of chemicals and products (Dean et al. 2001; ICCVAM 1999b; Sailstad et al. 2001).

Impact of New Technologies on Regulatory Testing and Humane Endpoints

New testing methods for safety assessments are being developed that incorporate advanced technologies and greater understanding of disease mechanisms. Examples of new technologies that are affecting regulatory testing methods include the following:

- Molecular biomarkers (e.g., toxicogenomics, proteomics, metabonomics);

- Transgenic models;
- High-throughput technologies;
- Computational modeling;
- Noninvasive imaging/labeling techniques;
- In vitro cell/tissue models; and
- Quantitative structure activity relationship (QSAR) models.

Toxicogenomics, in particular, is a promising science in which cellular differences in gene expression responses to toxicants are being evaluated as possible predictors of potential toxicity (NIEHS 2001a). These measurements are accomplished using microarray technology that allows for thousands of genes to be evaluated on one small glass slide. This technology should lead to improved understanding of the similarities and differences in human and animal responses to toxicants and facilitate our understanding of the relevance of animal studies to human health. Such information is also likely to support the development of mechanism-based biomarkers that can be used as earlier, more sensitive indicators of toxicity.

Summary

Humane endpoints have been developed and adapted for some types of regulatory testing that reduce the duration and, in some cases, the severity of unrelieved pain and distress in animals when pain-relieving agents cannot be used. Future progress in the development and successful implementation of humane endpoints will require the cooperative efforts of the entire testing community including scientists, regulatory staff, veterinarians, and animal technicians. The most significant progress will likely evolve from incorporating earlier biomarkers of adverse effects into testing methods and strategies. The development, validation, acceptance, and use of more humane endpoints in regulatory testing procedures will contribute to improved animal welfare while providing continued protection of human health.

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²Abbreviation used in this presentation: LLNA, local lymph node assay.

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