2009

Resolving Animal Distress and Pain: Principles and Examples of Good Practice in Various Fields of Research

Alicia Karas
Tufts University

Matthew C. Leach
Newcastle University

Karl A. Andrutis
University of Florida

Kathleen Conlee
The Humane Society of the United States

John P. Gluck
University of New Mexico

See next page for additional authors

Authors

Alicia Karas, Matthew C. Leach, Karl A. Andrutis, Kathleen Conlee, John P. Gluck, Andrew N. Rowan, and Martin L. Stephens

Resolving Animal Distress and Pain: Principles and Examples of Good Practice in Various Fields of Research

Alicia Karas, DVM[1], Matthew C. Leach, PhD[2], Karl A. Andrutis DVM, MS, DACLAM[3], Kathleen Conlee, BS, MPA[4], John P. Gluck PhD[5,6,7], Andrew Rowan, DPhil[4], Martin L. Stephens, PhD[4]

[1]Tufts University,[2]University of Newcastle,[3]University of Florida,[4]The Humane Society of the United States,[5]University of New Mexico,[6]Kennedy Institute of Ethics,[7]Georgetown University

Pain and distress are central topics in legislation, regulations, and standards regarding the use of animals in research. However, in practice, pain has received greatly increased attention in recent years, while attention to distress has lagged far behind, especially for distress that is not induced by pain. A contributing factor is that there is less information readily available on distress, including practical information on its recognition, assessment and alleviation.

This chapter attempts to help fill that void by reversing the usual pattern and giving greater attention to distress than to pain. In addition, we also bypass the pain versus distress dichotomy by adopting a holistic treatment of adverse effects, i.e., not parsing distress and pain, by providing guidance on how to assess deviations from normality through tools such as score sheets. Our aim is to provide practical information to IACUCs, scientists, technicians and animal care personnel.

We organize the chapter according to specific research areas and case studies. However, the principles and approaches are readily generalized to other research areas.

Karas begins by examining surgical competence and the impact of variation in surgical skill on post-surgical pain and distress, as well as on research outcomes. She discusses the surgical stress syndrome and ways it can be diminished. Drawing on the field of surgery in human medicine, Karas also provides recommendations on the various ways that surgical skills can be evaluated and enhanced, and that surgical outcomes can be improved.

Leach discusses his use of aversion studies to sort out the controversy over the use of carbon dioxide as a euthanasia agent in mice and other animals. His findings suggest that carbon dioxide alone is not an appropriate euthanasia agent. More generally, Leach indicates that indicators of pain and distress must be selected and interpreted with care, to avoid generating equivocal results and endless controversy. His work also demonstrates that valuable information on pain and distress can be obtained without causing more than momentary pain or distress to research animals.

Andrutis tackles the difficult issue of how to address distress in infectious disease models—both acute and chronic. He highlights the importance of understanding how the disease will likely affect the animal, by gathering data on clinical signs and behavior, including activity patterns, with the aid of tailored score sheets and measurements such as body temperature and weight. The key approach to amelioration of adverse effects consistent with study aims is the determination of humane endpoints and their implementation. Andrutis concludes that the pace of progress in refining infectious disease research has been slow. We hope his contribution to this chapter will encourage an expansion of this effort, both for the sake of animal welfare and research quality.

Conlee discusses ways in which the pain and distress associated with polyclonal antibody production can be minimized. Key issues include the choice of adjuvant, adjuvant volume, and injection sites, as well as the use of booster injections. This case study offers a good example of how seemingly minor refinements can greatly reduce pain and distress associated with commonly used procedures.

Gluck provides information on how to address complex issues associated with animal research on psychopathology, using anxiety models as his frame of reference. Psychopathology research on animals can often induce adverse states associated with distress, such as anxiety, fear or depression. Like research on pain, the challenge becomes how to mitigate the impact of effects one may be seeking to induce, maintain, and study. This poses a clear challenge to those who must review or carry out psychological research, or care for the animals in such studies. Gluck poses probing questions that should be asked during development and review of these research models.

Conlee, Stephens, and Rowan summarize a workshop held on the neglected field of refinement in toxicity testing. Production of guidelines regarding humane endpoints in toxicity testing (including for both acute and chronic testing) was a main goal of the workshop. Experts provide information on how to monitor animals used in toxicity testing for pain and distress, such as use of telemetry, clinical signs, and gene expression, as well as the use of postmortem pathology in order to determine humane endpoints for future study. Specific aspects of toxicity testing are also addressed, such as dosing volume and routes and frequency of administration. Not only is current knowledge assessed, but future areas of research are identified as well.

Finally, Appendix A provides useful references in terms of addressing pain and distress caused by certain areas of research as well as common procedures.

Effect of surgical technical skill on pain and distress in animals

Alicia Karas, DVM
Tufts University

In the absence of analgesics, surgical tissue manipulation under anesthesia typically causes pain upon awakening or shortly thereafter. Pain can last a day or less, or can continue in some cases for months to years (Perkins and Kehlet 2000). Although it seems reasonable to assume that the tissue handling skills of the surgeon have a major effect on the degree of pain caused by a given surgery, there has been seemingly little direct study of the impact of surgical skill level or the extent of surgical training on postoperative pain in any species. Yet, many of us who have seen the same painful procedure performed by both a novice and by an experienced person (such as intravenous catheter placement, or ovariohysterectomy in a veterinary school training laboratory), have noted there are often dramatic differences in the amount of pain associated with the same surgeries performed by those with different levels of surgical experience.

In laboratory animal research, the individuals who perform surgery may have varied levels of training, ranging from no prior experience to postgraduate surgical specialty competency. In larger animal species such as dogs or swine, especially in the case of relatively complicated procedures, surgery will often be performed by individuals with a significant amount of training. However, the typical investigator using rodent surgery models (for example, craniotomy, ovariohysterectomy, catheter or telemeter implantation) is unlikely to have come to the laboratory with any significant training or background in performing surgical techniques. In

addition, those experienced with surgery in one species (i.e. goat or human) may find that tissue handling requirements are very different in another species (i.e. mouse), and this difference may be problematic. The extent of training of investigators in animal manipulations is of considerable importance if pain and distress are indeed greater in animals subjected to less skillful handling or surgery.
In the absence of direct evidence that surgical skill level influences the amount of pain or distress an animal feels post-operatively, it is possible to approach the subject by examining three areas of information:

- the impact of degree of surgical trauma on pain and other morbidities,
- surgical training of MDs, and
- outcome measures.

Trauma and surgery directly cause pain, but the neural input of painful stimuli also initiates a series of hormonal and metabolic changes known as the "surgical stress response" which may further contribute to both pain and morbidity. General anesthesia and surgery activate an endocrine state of catabolism (stress response), the extent and duration of which correspond positively to the magnitude and duration of the procedure and which culminates in loss of body weight and muscle mass. At the same time, certain alterations in the state and function of the immune system occur (Desborough 2000). Tissue injury, including surgical wounds, trauma, and sepsis, can result in profound effects on the patient's immune function – principally via increases in circulating cytokine levels. Cytokines are plasma proteins produced by cells of the immune system. They play major roles in mediating inflammation in the damaged tissue as well as in modifying immunocyte surveillance and wound healing. Certain cytokines are intricately intertwined with the induction of the painful state, in that cytokines play a role in pain transmission, transduction, and perception of pain signals (Shafer 2003). Cytokines are also thought to be responsible for the feelings of malaise and for fever after surgery as well (Sheeran and Hall 1997).

Although believed to be strategically adaptive for the animal, the surgical stress response and the production of the cytokine response can be manipulated in a number of ways to decrease its magnitude, as excessive cytokine responses are believed to contribute to organ dysfunction and acute and chronic pain after surgery (Kehlet 2000, Beilin et al. 2003). Attempts to reduce the magnitude of stress and cytokine responses are aimed principally at reducing morbidity (in studies of human postoperative complications the improvement in outcome includes such measures as length of hospital stay or incidence of postoperative ileus, pneumonia, myocardial ischemia). Effective cytokine response reduction methodologies work by preventing pain, modifying fear and anxiety states, and reducing the invasiveness of the surgical procedure (Kehlet 2000, Kiecolt-Glaser et al 1998, Beilin et al 2003, Shafer 2003). Good examples exist of the positive effects of reducing pain.

Decreased immune surveillance after cancer surgery appears to enhance the metastatic potential for cancers in both humans and in animal models. Interventions that inhibit pain, such as administration of morphine, or spinal blockade, have been shown in many animal studies to reduce uptake and retention of injected tumor cells (Beilin et al 2003, Ben-Eliyahu et al 1999, Bar-Yosef et al 2001). The exact relationship between pain, immune function and administration of analgesics is currently the subject of intense investigation, particularly in the field of surgical oncology.

Minimization of tissue trauma reduces post-surgical pain, and it is generally accepted that reducing the size of the incision reduces the amount of pain felt by the patient. In smaller patients or animal subjects, incision length is often described as "small", thereby implying that it

is less consequential to the patient. However incision size should be considered as proportional to body size, so in small patients incisions are often relatively large in order to facilitate access to the surgical site by comparatively large fingers and instruments. *Minimally invasive surgery* is an emerging field, gaining popularity for the reduced pain, convalescence, and severity of impact upon the patient that it apparently causes. Rather than "open" techniques which involve an incision that permits manual manipulation, and thus large amounts of surgical trauma, in minimally invasive surgery, manipulations are performed by means of small bore rigid or flexible instruments (laparoscopes, endoscopes) inserted through small stab incisions. Again, the relationship between pain, tissue damage and immune function is important here. Minimally invasive techniques are widely cited to decrease postoperative pain in both humans and animal models (Walsh et al 1999). A model to study standard versus minimally invasive laparotomy techniques in mice showed correspondingly lower production of certain deleterious cytokines in the mice having the less invasive procedure, thereby lessening the degree of immunosuppression (Iwanaka et al 1997).

Chronic pain states are documented to result from a number of major surgical procedures in humans, including amputation of limbs, thoracotomy, mastectomy, and hernia repair. Chronic pain states may develop in up to 50 – 81% of such surgeries (Perkins and Kehlet 2000). A number of perioperative factors were found to predict the generation of a chronic pain state: these include pre-existing pain, repeat surgery, surgical techniques where nerve damage might occur, acute postoperative pain, and anxiety (Perkins and Kehlet 2000). This indicates that methods to combat acute pain and reduce anxiety, as well as methods to reduce the extent of surgical trauma, could ostensibly reduce both short and long term pain in animals.

The literature about teaching / learning of surgical skills by MD trainees appears to support the premise that complication rates and duration of surgery are related to the number of procedures performed and the quality of surgical training. In other words, practice makes a competent surgeon, reducing procedure duration and improving outcome. Weise et al. (2004) examined complication and outcome rates for a surgical procedure dependent on the level of surgical training, based on number of cases performed prior to the assessment. This study found that more experienced surgeons (those having performed 10 times the number of surgeries compared to the less experienced group) had a complication rate of 2.2% versus 10.7% for the less experienced group. The investigators noted that some of the recorded complications were associated with greater initial postoperative pain. Georgeson and Owings (2000) cited the inverse relationship between duration of procedure and surgical experience (number of surgeries performed) in MD surgeons; when surgeons had performed fewer than 10 of a particular endoscopic procedure, the duration of that surgery was roughly double that of surgeons who had performed the same surgery 40 times. If surgical time increases postoperative pain due to increased tissue handling, then one might surmise that as surgeons move along the learning curve for a technique, pain from a surgery will be reduced.

In studies on the effects of surgery on mice in my own laboratory, we found that the average 24-hour post-operative weight loss in a study where the surgeon was a presumably competent graduate student was 12%. However, in a follow-up study where the surgeon was very experienced, the average 24-hour post-operative weight loss was just 7%; this points to the possibility of tracking not just the duration of a specific surgery but also post-operative weight loss as a measure of surgical skill.

It is not surprising that both the quality of training and a surgeon's innate visuospatial and psychomotor skills are essential components of surgical proficiency (Khan et al, 2003). Keeping in mind that the studies cited in this review center upon MDs with at least several years of

postgraduate training in surgery, it is important to consider whether performing a surgery 5 to 10 times leads to sufficient skill in a novice investigator with little to no background in anatomy or surgery. This leads to the question of how competency in laboratory animal surgery should be acquired and assessed. Technical surgical skill is fundamental to both accurate science and humane science if skill correlates with outcome, producing a viable and useful experimental model, and if skill correlates with degree of postoperative pain. If, for example, the learning curve is steep over the first 10 – 20 surgeries, then lack of skill in the investigator-surgeon will be expected to introduce a significant experimental treatment-order bias. Models for training and evaluating surgeons can include actual patients, surrogate patients (live animals), cadavers, and inanimate models. Datta et al. (2004) concluded that assessments of technical skill in surgical trainees using inanimate (bench top) models correlated well with actual surgical performance on humans, using a structured system of evaluation, the "objective structured assessment of technical skill" or OSATS. No comparable system of tracking or assessing competency of individuals performing surgery on laboratory animals is currently advocated. If, however, a uniform animal subject population is felt to be critical to experimental investigation, then the development of methods of assessment of surgical skill as well as methods to subjectively evaluate animals/outcomes after surgery are important in order to detect and prevent unwelcome variation in experimental models.

Taking all of the above into account, it can be argued that the end result from a surgical procedure in a laboratory animal model may depend significantly upon the degree of pain or invasiveness of a procedure, which may depend upon the skill of the surgeon, which in turn depends upon the quality and quantity of training, as well as upon innate characteristics of the surgeon. Scientists frequently balk at the introduction of new methods, such as anesthesia, analgesics, or environmental enrichment to prevent distress, worried that these methods increase the risk of variability, potentially making the experimental results less valid, unable to be replicated, or less acceptable by peer review. A case can be made therefore, that inadequate surgical skill, or unevenness in the exposure of animals to handling by a surgeon on the steep portion of a technical learning curve, has the potential to adversely affect research results in a similar way.

It is essential for scientists to be aware of the effects of surgery (pain, immunomodulation, endocrine responses) on animals. To avoid variability in results, there is a need for a general recognition of the effects of skill level on outcome, to develop methods to evaluate and track surgical skill level, and to more carefully monitor animals to detect when surgical pain or distress are above what is considered to be normal or anticipated, given the manipulation. Such a refinement of scientific method might also be expected to reduce pain and distress in animal subjects, particularly if training on procedures makes use of inanimate models or simulations and cadavers as has proved effective. Investigators would then proceed to survival surgeries only when a certain level of competency is reached.

Carbon dioxide euthanasia: example of aversion techniques

Matthew C. Leach, PhD
University of Newcastle

Carbon dioxide exposure is a recommended and widely used method of euthanasia for many laboratory and farm animal species (Blackshaw et al., 1988; Blackmore, 1993; Coenen et al., 1995; EU Working Party, 1996, 1997; Danneman et al., 1997; Kohler et al., 1999; van Luijtelaar & Coenen, 1999; Hackbarth et al., 2000). It is considered by many regulatory organizations (e.g. AVMA 2000; Home Office 1997) to fulfil the criteria for an effective agent of euthanasia, as

it is cost effective, readily available, aesthetically acceptable to humans, and can be used to kill many animals simultaneously. However, questions remain over whether carbon dioxide can induce unconsciousness and death humanely, which is one of the most important, and yet often forgotten, characteristics of agents of euthanasia. To be considered humane, an agent must minimize any suffering associated with induction to unconsciousness, i.e. animal's initial perception of an agent, the pain and distress that is experienced before loss of consciousness, and the time taken to induce unconsciousness. Although carbon dioxide induces a rapid loss of consciousness at concentrations above 40% (Coenen et al., 1995; Kohler et al., 1999; van Luijtelaar & Coenen, 1999) and death above 70% (Iwarsson & Rehbinder, 1993), its ability to induce unconsciousness humanely remains extremely controversial.

The controversy surrounding carbon dioxide stems from the contradictory findings of the previous studies assessing the humaneness of carbon dioxide. Some authors have concluded that carbon dioxide concentrations high enough to cause loss of consciousness and death provide a non-distressing induction for both rodents (Blackshaw et al., 1988; Hewett et al., 1993; Smith & Harrap, 1997; Kohler et al., 1999; Hackbarth et al., 2000) and other species (Mullenax & Dougherty, 1963), whereas others have concluded that such concentrations cause considerable distress before loss of consciousness in both rodents (Iwarrson & Rehbinder, 1993; Coenen et al., 1995; Ludders et al., 1999) and other species (Lucke, 1979; Raj & Gregory, 1994; Raj & Whittington, 1995; Raj & Gregory, 1995; van Luijtelaar & Coenen, 1999). The contradictory nature of these studies seems to relate to the fact that they used only a limited number of essentially the same behavioral measures to assess aversion, and that there were considerable differences in the interpretation of these behaviors between studies (Danneman et al., 1997), leading to a diversity of conclusions regarding their implications for welfare.

The potential distress associated with carbon dioxide has been attributed to irritation of the nasal mucosal membranes (Lucke, 1979; Ewbank, 1983; Iwarrson & Rehbinder, 1993) and hypoxia and hypercapnia causing breathlessness and hyperventilation (Hewett et al., 1993; Raj & Gregory, 1995; Raj & Whittington, 1995; Lambooij et al., 1999; Ludders et al., 1999; van Luijtelaar & Coenen, 1999). As carbon dioxide exposure is considered by many to offer an economic and rapid euthanasia technique, a number of modifications have been suggested to reduce these potential problems. First, both placing animals into a rising verses static concentration of carbon dioxide (Kohler et al. 1999), and introducing carbon dioxide after animals have been placed into a chamber, have been suggested to induce the loss of consciousness before animals are exposed to higher concentrations that may associated with pain and distress. Secondly, humidification has been suggested to reduce irritation resulting from the dry nature of the gas (MacArthur, 1978; Mouton et al., 2001). Thirdly, the addition of exogenous oxygen has been suggested to reduce the level of potentially distressing hypoxia experienced before loss of consciousness (Anon, 1967; Iwarsson & Rehbinder, 1993; Coenen et al., 1995; EU Working Party Report, 1996; Danneman et al., 1997; Smith & Harrap, 1997; Kohler et al., 1999). Finally, combinations of low carbon dioxide (30%) and high argon concentrations (60%) have been tested recently with farm animal species. This combination is thought to be as efficient as and more humane than carbon dioxide alone (Raj & Gregory, 1994; Raj & Whittington, 1995; Raj, 1999), as it is suggested to cause unconsciousness and death by hypoxia without causing the breathlessness and painful irritation of the mucous membranes (Raj & Gregory, 1994; Lambooij et al., 1999; van Luijtelaar & Coenen, 1999; Raj & Whittington, 1995; Raj, 1999).

More recently the work of Leach et al., (2002a,b; 2003) has attempted to untangle the controversy surrounding the humaneness of carbon dioxide and to assess the extent of

aversion associated with it, compared to the potentially more humane modifications and other alternative gaseous euthanasia agents. These studies assessed aversion of laboratory rodents to carbon dioxide (humidified & non-humidified), argon, carbon dioxide-argon mixtures, carbon dioxide-oxygen mixtures, and volatile liquid anesthetics using a wide range of aversion measures in an attempt to gain a clearer insight into what an animal might experience, as opposed to what a human might interpret by observing animal behavior. These studies used measurements of an animal's attempts to escape from and avoid the potentially noxious agents (dwelling time, numbers of occasions of withdrawal and re-entry), alongside the behavioral measures used to assess rodent aversion in previous studies. The results of this work have demonstrated that the behavioral measures that have formed the basis of almost all previous aversion studies are very poor measures of aversion, and therefore cannot be relied upon to effectively measure the animal's reactions (aversion) to the agents. This helps explain why the results of these studies are so contradictory.

Alternatively, the measurements of the animal's attempts to escape or avoid the noxious stimulus proved to be considerably more effective measures of the animal's reaction. These measures demonstrated that carbon dioxide caused considerable aversion and that an animal confined in an environment containing these gases is likely to suffer considerable pain and distress before unconsciousness occurs. Carbon dioxide caused significant aversion whether presented alone (humidified & non-humidified) or in combination with oxygen or argon (even at a low concentrations sufficient to induce a loss of consciousness, let alone those high enough to cause death). Most importantly, aversion was observed with these agents at very low concentrations, which are below those that will effectively induce unconsciousness and are likely to be reached very rapidly even with a slow rising concentration, as tested in some of the other studies (Fenwick & Blackshaw 1989; Kohler et al. 1999). The remaining agents appear to be far less aversive than carbon dioxide and its combinations, with the volatile liquid anesthetics proving to be by far the least aversive agents tested. Argon induced aversion at a level between that of the anesthetics and carbon dioxide.

The finding that carbon dioxide may cause considerable pain and distress before unconsciousness should perhaps not be surprising, as human and animal pain research has routinely used carbon dioxide as a noxious stimulus at similar concentrations to those recommended for euthanasia (Thurauf et al., 1991; Anton et al., 1992; Komai & Bryant, 1993; Peppel & Anton, 1993; Danneman et al., 1997). This begs the question: how can an agent induce unconsciousness humanely and be a noxious stimulus at the same concentration? Humans report carbon dioxide exposure at levels sufficient to cause unconsciousness in animals (above 40%), as 'unpleasant and distressing'; levels sufficient to kill (above 70%) are described as 'painful' (Paton 1983; Gregory et al. 1990). Based upon the current understanding of comparative anatomy and physiology of respiration and pain (Raj et al., 2004), and the fact that animals exhibit signs of aversion at similar concentrations to those of humans, it seems extremely likely that they experience similar distressing and painful sensations on exposure to carbon dioxide. Therefore, carbon dioxide should not be used for euthanasia by this principle alone.

The results of existing papers directly investigating the humaneness of carbon dioxide euthanasia in laboratory and farm animal species, and those that have assessed carbon dioxide exposure on human subjects, demonstrate that carbon dioxide in the form/s that it is currently used cannot induce unconsciousness, let alone death, without causing considerable degree of pain and suffering. A viable alternative to using carbon dioxide alone would be to use a volatile liquid anaesthetic (e.g. halothane or enflurane) in order to induce unconsciousness and then to

subsequently kill the animals rapidly with carbon dioxide once the animals are unconscious (Leach et al., 2002a,b; Leach et al., 2003; Raj et al., 2004).

The Refinement of Infectious Disease Research

Karl A. Andrutis DVM, MS, DACLAM
University of Florida

Infectious disease research on animals can cause varying degrees of pain or distress as a consequence of exposing (challenging) the animals to infectious agents or their toxins and letting the resulting morbidity and mortality ensue. Everyone presumes that a sick animal is in a state of reduced welfare but why does sickness produce a negative state? Gregory (1998 & 2004) and Maier and Watkins (1998) link the status of the immune system and "feeling" states in animals. According to Gregory (1998), there are various "sickness behaviors" or responses that are common to a wide variety of diseases. These include fever, hyperalgesia, reduced movement and appetite, social isolation, muscle catabolism and pain, and impaired memory and learning. Behaviors such as isolation and rest intuitively would help prevent the spread of disease and promote healing while fever and fasting appear to suppress the pathogenicity of some micro-organisms.

The behavioral responses have a physiological basis and are linked to the activity of certain cytokines – especially interleukin-1 (*IL-1*), interleukin-6 (*IL-6*) and tumor necrosis factor (*TNF*). Maier and Watkins (1998) report that blocking the relevant cytokines prevents the characteristic sickness behaviors while injecting the same cytokines into healthy animals produces the full syndrome of responses. IL-1 is the most potent in producing these behaviors and is strongly induced by the endotoxin, lipopolysaccharide. The cytokines do not appear to cross the blood-brain barrier and their effects on the CNS seem to be mediated in part by the vagus nerve.

Kelley & Dantzer (2007), in a recent review, note that the newly-defined role of cytokines in a wide variety of systemic co-morbid conditions, ranging from chronic heart failure to obesity, may begin to explain changes in the mental state of such subjects. They continue that there are a number of pharmacological tools available to antagonize the detrimental actions of cytokines.

Infectious disease research would seem to be an obvious opportunity for applying alternative methods especially refinements; however; progress in this area has been slow. In this section, we briefly summarize the literature on refinement in infectious disease research, emphasizing the use of tailored score sheets and humane endpoints. Other approaches, such as the use of sophisticated noninvasive imaging techniques that limit both animal suffering and sample sizes, are reviewed elsewhere (Contag, C.H. et al, 1995; Contag P.R., et al., 2008).

As stressed by previous reviewers of refinement in infectious disease studies (Hamm 1995, Olfert and Godson 2000), the key ingredient to implementing effective monitoring strategies and humane endpoints is a cooperative, and possibly collaborative, working relationship between the principal investigator, the veterinary and animal care staff, and the Institutional Animal Care and Use Committee (IACUC) (Medina 2004).

Although the consideration of alternative methods prior to performing animal studies is mandated by regulatory authorities (Public Health Service 2002, United States Department of Agriculture 2002), many scientists continue to view this requirement as intrusive and not beneficial to their work. Those scientists that have embraced the concept of alternatives view the development, evaluation, and implementation of alternative methods as identical to the

refinements made in any scientific endeavor in which methods are modified to produce the highest quality results.

The suggested approach to the development, evaluation, and implementation of alternatives in infectious disease research begins with resolving the philosophical differences regarding alternatives or, at least, coming to some agreement that refinement of animal use is compatible with good science and may, in fact, lead to better results. Once the principal investigator agrees that developing alternative methods is a worthy goal, the alternatives-related work can begin.

The investigator should understand the pathogenic mechanisms by which the infectious agent or toxin acts on the animal. This understanding will assist in the identification of clinical signs or other physiological and behavioral parameters that should be monitored during the course of the study. The time course of pathogenesis will be critical to determine the logistical aspects of dosing and monitoring of the animals. Without this understanding, one needs to start with less specific indicators such as body weight, body condition score (Ullman-Cullere and Foltz 1999), activity levels and other simple behavioral assessments to identify criteria that would be indicative of morbidity. A good example of a relatively non-specific clinical sign to assess pain or distress is the recently described method for assessing stress in rats by scoring chromodacryorrhea (secretion of porphyrin-filled tears—commonly referred to as “red tears” or “bloody tears”) (Mason et al., 2004). Evaluation of activity patterns has been a useful parameter in infectious disease (Olfert and Godson 2000, Vlach et al. 2000) and other models (Karas et al. 2001).

One of the best ways to record and organize these assessments is by the use of a score sheet or monitoring form (Morton 2000, van der Meer et al. 2001). Development of a simple yet complete monitoring form for a given type of research is critical to the success of identifying determinants of a humane endpoint. The ease of use of the monitoring form will determine investigator compliance as well as the ability to interpret the data. Effort should be made at the beginning of the study to predict which parameters to assess and the method of scoring these parameters. The parameters and the scoring system may need to be modified as the study progresses and new information is obtained. Research and animal care staff must be trained to understand the clinical signs or other parameters being assessed and the basis of the scoring system. Review of the data collected on monitoring forms should occur frequently to assess its value and interpretation.

A more complete understanding of the pathophysiology involved in the animal model might identify more specific parameters for monitoring animals. Studies involving acute infections or toxicity may require assessment of different parameters than models of chronic infections. Whereas weight loss and body condition score might be very useful parameters in a chronic model, they may be less useful or unchanged in an acute model. Assessment of particular organ systems may require training and skill in collecting samples for laboratory measurement and assessing clinical signs such as respiratory rate or effort. Remote monitoring of physiologic variables such as heart rate, blood pressure, and body temperature may also be performed with telemetry (Morton et al. 2003), implantable microchips, or infrared thermometer. These are additional refinements that could be used, though they come at the “cost” of any pain or distress associated with their implantation and maintenance in the animals’ body.

Body temperature has been used as a possible determinant of humane endpoints (Toth 2000, Olfert and Godson 2000). Some models of infectious disease have found body temperature to be predictive of mortality (Wong et al. 1997, Kort et al. 1998, Vlach et al. 2000, Warn et al. 2003) while body temperature was found not to be a useful parameter in other models (Krarup

et al. 1999). Furthermore, body temperature may or may not be altered during certain periods of an infection and must be assessed at the appropriate time during the course of the study. Other parameters may also vary during the course of infection and the timing of assessments may be critical to their value in identifying a humane endpoint.

As mentioned, a thorough understanding of the pathophysiology of the animal model, determination of the parameters to be monitored, and the scoring system used are of critical importance in the process of developing humane endpoints. The real question in the search for a humane endpoint is the proximate cause of death. Many investigators believe that the cause of death is directly related to their experimental manipulation such as infection or toxicity. But this assumption is not enough to define a cause of death. Perhaps the cause of death is sepsis with its many physiological consequences ultimately resulting in shock and cardiovascular collapse. Or perhaps cardiovascular collapse is the result of prolonged inappetance and profound dehydration as a secondary effect of the infectious process, i.e., the sickness behaviors discussed earlier. Since most animal models do not include supportive care during the course of infection, this distinction may be very important, particularly in therapeutic studies where subtle differences may be critical. Identifying the proximate cause of death should assist in monitoring the critical parameters leading to morbidity and mortality.

Davis has provided a case study of how to relieve symptoms of pain and distress in a disease model, experimental allergic encephalitis (EAS), through palliative care while also facilitating achievement of study objectives (Davis, 1999/2000). The investigator initially proposed a grading scale of EAE obtained in the published literature. This proposed scale simply indicated a grade that corresponded with specific clinical signs, with no mention of intervention. For example, the clinical signs for grade 3 were indicated as “partial hind limb paralysis,” with no mention of intervention/care.

The veterinary staff then met with investigators to develop a mutually acceptable grading scheme that would meet study objectives, establish guidelines for intervention, and would not interfere with study goals. As a result, the clinical signs for grade 3 included “moderate paraparesis: inability to move one or both hindlegs, possible atonic bladder, noticeable gait disturbance.” Additionally, the intervention was specified as “food and water more accessible (feed pellets and fruit placed on floor of the cage), express urinary bladder, if needed, weigh 3 times weekly, euthanize if $\geq$ 20 percent weight loss.”

As observational skills developed, they began categorizing mice in EAE grade 1 earlier and earlier, which resulted in more intense monitoring and nursing and a modified assessment chart. For example, the final grading scheme indicated clinical signs for grade 3 as “moderate paraparesis: inability to move one or both hindlegs, noticeable gait disturbance, possible atonic bladders.” The corresponding intervention was “food and water more accessible (for example, feed mash placed on floor of cage, water bottle w/long sipper tube, and fruit as fluid supplementation). Express urinary bladder twice daily; give fluids, if necessary. Animals may need supplemental heat. Weigh at least three times per week. Euthanize if $\geq$ 20 percent body weight loss.”

The outcomes of this grading scheme effort were

- Improved assessment and alleviation of animal pain and distress
- Animals who lived longer, which allowed investigators to reach study endpoints.
- Requests from the investigators for the observational data (i.e. weight) to correlate with their measurement of disease.

The development, evaluation, and implementation of alternative methods in infectious disease research requires the cooperation of the principle investigator and research staff, the veterinarian and animal care staff, and the IACUC. The process of developing humane endpoints while meeting the scientific objectives of the research is dependent on the committed involvement of these individuals. Key personnel ingredients for success include adequate training (to identify the appropriate parameters to monitor) and sufficient dedication to carry out monitoring at the appropriate time (often occurring after normal work hours). The careful evaluation and interpretation of the monitoring data and its association with the time course of morbidity and mortality in the study will allow for improvements in refinement alternatives in infectious disease research.

Polyclonal antibody production

Kathleen Conlee, BS, MPA
The Humane Society of the United States

Polyclonal antibody (Pab) production has significant potential to cause animal pain and distress. However, this pain and distress may not be appropriately addressed because antibody production is only one procedure within a larger research objective, such as their application to vaccine quality control, diagnostic testing, cancer therapies, or immunological research. There are a number of steps involved with Pab production itself that likely cause pain and distress, including choice and volume of adjuvant, injection site, blood drawing, and use of booster injections.

Production of antibodies in a human or nonhuman is achieved by injecting an adjuvant (antigen) that is recognized as foreign to the body in order to stimulate the immune system. Monoclonal antibodies are produced by cells that are derived from a single clone of an antibody-producing cell, whereas Pabs are derived from many different clones that will bind to many sites on the antigen; therefore they have decreased specificity in comparison with Mabs, but a range of differing avidities (i.e., strength of binding). Researchers seek to maximize antibody yield. This aim can be viewed as in conflict with animal welfare, but this does not need to be the case.

In order to address minimization of pain and distress associated with the various steps of Pab production, The Humane Society of the United States (HSUS) convened a workshop of international experts[1] in the fields of antibody production, animal welfare, *in vitro* alternatives and/or regulatory compliance. The expert recommendations resulting from the workshop are summarized here (the entire manuscript from the workshop can be found on The HSUS website at http://www.hsus.org/animals_in_research/pain_distress/pain_and_distress_associated_with_polyclonal_antibody_production.html) and a sample score sheet for the assessment of animals used for polyclonal antibody production is included as well (see Table 1). Information that has

[1] Workshop participants (and affiliation at that time) included: Vera Baumans, Ph.D. Karolinska Institute; Kathleen Conlee, M.P.A., The Humane Society of the United States; Wim A. deLeeuw, D.V.M., Ministry of Public Health in the Netherlands; Coenraad Hendriksen, D.V.M., Ph.D., Netherlands Vaccine Institute, Netherlands Center for Alternatives to Animal Use; David Johnson, D.V.M., advisor for Harlan, Inc.; John McArdle, Ph.D., Center for Alternatives Resources; David Morton, B.V.Sc., Ph.D., University of Birmingham; Norm Peterson, D.V.M., Johns Hopkins University; Jon Richmond, M.D., Ph.D., Home Office, United Kingdom; Margaret Rose, Ph.D., University of New South Wales; Andrew Rowan, Ph.D., The Humane Society of the United States; and Harold Stills, D.V.M., Wright State University School of Medicine.

become available since the expert workshop will be discussed in this section as well. Importantly, the Institute for Laboratory Animal Research Journal devoted an issue to immunization procedures and adjuvants (*ILAR Journal* Volume 46, Number 3, 2005).

Before the workshop participants tackled specific procedures and techniques involved with antibody production, all agreed that it was first important to emphasize that only experienced personnel should be allowed to engage in Pab production. Personnel must be able to assess the animals for distress and pain and handle animals appropriately--a general rule should apply to all procedures involving the use of animals.

A. The workshop participants recommended utilizing the chicken egg yolk (IgY) technique for Pab production when possible. Polyclonal antibody production most commonly involves the use of rabbits given the relative ease of housing, handling and taking blood from these animals; however, guinea pigs, rats, large mammals, and others are used as well (Schade et al. 1996). The use of chickens, however, is considered a valuable alternative for a number of reasons.

First, Pabs are produced in chicken egg yolk and significantly larger quantities of antibodies are produced per chicken in comparison to other animals, which reduces the number of animals needed for antibody production. Furthermore, the egg yolk technique (also referred to immunoglobulin Y, or IgY, technique) does not require bleeding of the chickens, an invasive technique used when producing antibodies in other animals. The avoidance of this step can greatly reduce distress associated with Pab production (Schade et al. 1996). Also, the sites at which adjuvants are injected do not appear to have the marked local inflammatory response as is often seen in mammals. Finally, false positive reactions in certain immunchemical assays are unlikely. For the various welfare and scientific reasons discussed here, the use of the chicken egg yolk technique for Pab production should be utilized whenever possible.

One main reason that many institutions do not use chickens, despite the associated advantages, is the lack of facilities to properly care for chickens. In this case, institutions should opt for outsourcing Pab production using the chicken egg yolk technique instead of carrying the procedures out in-house. Of course, only qualified and reputable commercial suppliers should be used. For those institutions that have Public Health Service (PHS) assurance, the supplier must be PHS-assured as well.

B. It was agreed that oral immunization is preferable over injections routes. According to Hau and Hendriksen (2005) there are now more oral immunization choices available so that injection is not necessary; these include aerosol or voluntary oral intake

C. Participants agreed that intramuscular (IM), intraperitoneal (IP), intrasplenic,
intravenous (IV), and footpad injections should be discouraged. If these routes are used, strong scientific justification should be provided.

Adjuvants that can be coadministered with antigens orally and nasally are new developments (Eriksson & Holmgren, 2002; Foss & Murtaugh, 2000); researchers are encouraged to monitor the emerging literature in this area, as oral and nasal routes of administration may produce an appropriate antibody response and may be preferred in regard to animal welfare. A major problem is that a local immunity (IgA) response is elicited rather than a humoral (IgG) response.

D. If oral immunization is not used, subcutaneous (SC) and intradermal (ID) are considered to be acceptable routes of injection. There was some discussion and disagreement over which of these two routes is preferable.

E. The chosen adjuvant should ideally induce high antibody titers in serum and/or egg yolk while minimizing pain and distress. Adjuvant choice should be carefully determined and pilot studies are suggested as one way to determine the best adjuvant for a particular immunogen or class of antigens.

F. Recommendation: Freund's Complete Adjuvant (FCA) may not be as problematic with regard to pain and distress as has been suggested and may have advantages over other adjuvants when used properly. However, when using FCA, it is crucial that the volume used be minimized (based on route of injection and animal species), the sample for injection is properly prepared, and the administration competently performed. Under no circumstances should an animal be given a second injection of FCA.

G. Regardless of the adjuvant chosen, the smallest volume possible that produces an adequate antibody response should be used. It is recommended that very small volumes be injected into multiple sites for an enhanced antibody response.

Workshop participants also identified a number of areas in which further research is necessary:

- Determine species-specific information for welfare and pain and distress assessments (including behavioral data).
- Develop alternatives to adjuvants in order to create an immune response while decreasing animal pain and distress
- Create new adjuvants and immunization methods in order to decrease animal pain and distress.
- Determine what role pain plays in the antibody response.
- Examine the effects of varying amounts of mycobacteria in adjuvants in order to decipher preferable levels.
- Determine if cage enrichment and group housing would simplify recognition of welfare problems, increase animals' psychological well-being (Turner et al., 1997), and increase production of Pabs.
- Determine whether there are pathological effects indicative of pain and distress during Pab production.

It was emphasized that such research should, whenever possible, be "piggybacked" onto existing work in order to avoid the use of additional animals.

The amount of scientific information on Pab production is increasing at a rapid rate; consequently, personnel carrying out Pab production should keep up with the literature in order to reduce animal pain and distress associated with this common procedure.

Animal Models of Human Psychopathology: Anxiety

John P. Gluck Ph.D.
University of New Mexico
Kennedy Institute of Ethics, Georgetown University

Psychology is a broad and diverse scientific enterprise. As a consequence, the research topics that make use of animals are many and include basic studies of perception, learning, cognition, motivation, animal communication, behavior genetics, behavioral evolution, brain/ behavior relationships, development, and models of human psychopathology. Animal models of

abnormal emotional behavior such as anxiety disorders represent some of the most controversial uses of animals in psychology and will be the focus of this section. The purpose of this section is to provide the historical context of this research, familiarize the reader with the range of anxiety disorders diagnosable in humans and with a representative sample of the animal methods that have been used to study these disorders. The section concludes with a set of general questions which may be useful in facilitating a discussion between researchers and IACUC reviewers, with the intended outcome of refining procedures to limit animal distress and pain.

Psychology and the Use of Animal Models of Psychopathology

Early in the twentieth century the Nobel Prize winning physiologist Ivan Pavlov was studying learning mechanisms that determined how specific environmental stimuli came to be associated with specific behavioral responses. Research on these "conditioning" processes made extensive use of dogs, sheep, pigs and goats as experimental subjects. In the well-known prototypical experiment, a biologically neutral stimulus like a clicking metronome was paired with the insertion of food powder into the mouth of a dog. The food powder immediately elicited salivation. It was found that after a number of such pairings the sound of the metronome alone would call forth the salivation. In other paradigms a neutral stimulus was paired with electric shock to the forelimb of a goat. The shock would elicit a flexion of the leg, which would eventually be elicited by the neutral stimulus alone (see Pavlov, 1928).

However, in these types of conditioning experiments it was also frequently observed that if an animal was tested continually, over a period of months or even years, or was required to discriminate between different stimuli that were very similar, the associations would eventually breakdown and be replaced by what was referred to as "experimental neurosis". For example, instead of a simple leg flexion the animal might begin to show exaggerated defensive responses, rigidity, disturbed vocalizations, and desperate attempts to escape. These reactions were quite bizarre and were soon seen as challenging the Freudian perspective that suggested that the burden of mental illness was limited to human beings. Researchers like Gantt and Liddell (see Wolpe et al. 1964) published papers and gave live demonstrations of "neurotic" animals that were intended to advance the claim that these complex emotional disorders could be studied in animals. In other words, these observations led to a consideration that animals could serve as stand-in laboratory models for humans not only for physical diseases like infections and cancer but psychological conditions as well.

Later in the twentieth century researchers like Harry F. Harlow et al. (1971) and Martin Seligman (1975) added to the methods of creating psychopathology in animals by developing laboratory analogies of traumas purported to be crucial in creating abnormal behavior in humans. For example, Harlow separated monkey infants from their mothers in an attempt to replicate the human developmental deprivation defects emphasized by theorists such as the British psychiatrist John Bowlby (1988). Similarly, Seligman exposed animals to environments in which they were "helpless" to exert control on the delivery of aversive stimuli, thereby intending to create an analogy of the helpless experience of depression reported by some patients. This simulation or analogy approach is still dominant in the current literature on animal models. In addition, with the increase in the understanding about the contributions of genetics to mental disorders, animal models that involve developing strains of animals with the desired behavioral characteristic by selective breeding or by "knocking out" genetic material suspected to be relevant to the psychological condition have begun to appear.

Animal Models of Psychopathology and the IACUC

In general, reviews of protocols that involve animal models of psychopathology pose particular difficulties for the IACUC. First, there can be no question about the need for continued development of effective mental health interventions. Mental disorders affect many people during their lifetime, inflicting distress and disability that extract a great toll from the affected individuals and their families. Second, human biochemical, post- mortem, and brain imaging studies have contributed mounting suggestive evidence about the existence of identifiable changes in neurotransmitter availability and brain architecture in people with mental disorders. As a consequence, the current treatment zeitgeist in psychology and psychiatry has increasingly come to emphasize the use of pharmacological interventions to ameliorate the clinical manifestations. Therefore, research methods necessary for locating abnormal neural alterations, understanding the mechanisms of action of possible medications, and the screening of potential agents for effectiveness and safety prior to the start of human clinical trials, have gained priority. Third, the development and use of animal models of human psychopathology has a long history and tradition in psychology that results in a somewhat entrenched positive presumption about their reliability and usefulness. Fourth, the very purpose of the models is to produce distress to an extent that a serious psychological pathology can be said to have been created and then <u>maintained</u> for some extended period of time so that the goals of the experiments can be achieved. Consequently, issues concerning the application of the three Rs are not at all straight forward. Fifth, while the symptoms of human psychopathology often have some clearly observable behavioral changes (e.g. exaggerated startle response in Post Traumatic Stress Disorder, extreme avoidance of objects and places in specific phobias) diagnosis and treatment progress in humans is primarily based upon the reports of subjective experience of the patient. Therefore the degree to which an animal model actually maps on to the human condition is always to some degree in question.

Classification of Human Anxiety Disorders

It has been estimated that the prevalence of mental disorders in the human adult population alone is approximately 20 percent, with anxiety disorders accounting for over 16 per cent of the total (Regier et al., 1993; Kessler et al., 1994). Anxiety disorders in humans are manifested in a variety of different ways. The authoritative Data and Statistical Manual of the American Psychiatric Association (DSM IV TR) lists 11 separate anxiety disorders. All share, to varying degrees, a set of symptoms that includes intrusive thoughts, irrational fears, feelings of dread, behavioral compulsions, edginess, irritability, increased startle reactions, sleeplessness, heart palpitations, chest pain, sweating, headaches, and rapid shallow breathing. The basic disorders are described as follows.

1. <u>General Anxiety Disorder.</u> This disorder is characterized by high levels of worry about the issues of everyday life to an extent that functioning is impaired. Physical symptoms include headaches, weakness, edginess, sleeplessness, and irritability.

2. <u>Acute Stress and Post Traumatic Stress Disorder</u>. Acute stress disorder refers to the anxiety symptoms that develop within the first month after exposure to an extreme trauma such as a violent crime like rape, murder, assault, serious motor vehicle accident, and military combat. The symptoms include generalized anxiety, increased startle, social avoidance, and recurrent thoughts or flashbacks about the experience. If symptoms continue, post traumatic stress disorder is diagnosed when patients lose self-esteem, become cynical about life and relationships, feel permanently broken, and become vulnerable to substance abuse

3. <u>Obsessive Compulsive Disorder</u>. Patients experience unwanted intrusive and persistent thoughts that are perceived as inappropriate or even grotesque. Compulsions are demanding

repetitive behaviors (e.g. lock checking, hand washing) that reflect the patient's attempt to control their anxiety.

4. Panic Disorder with or without Agoraphobia. The central experience of a panic disorder is a very rapid onset of the physical symptoms described above coupled with fear of their recurrence. Agoraphobia refers to fear of situations where help or escape from a panic attack would be impossible or publicly humiliating.

5. Phobias. A phobia is an intense irrational fear of a specific object (e.g. snakes, heights) or social situation that results in avoidance. Social phobias tend to involve powerful fears of being examined, judged, rejected and embarrassed while in public.

Animal Models of Anxiety Disorders

Just as there are many types of human anxiety disorders, there are many approaches to creating and measuring anxiety in animals for the purpose of research. However the purpose of the models tends to be redundant rather than capturing the diversity of the human disorders. Unlike in the animal models used to study pain, researchers involved in anxiety studies have not explicitly addressed the ethical issues raised by their research. There has been little discussion in the literature of the merits of a test that allows the animal some level of control (e.g. the open field test) versus a model that forces the animal into an unpleasant situation from which there is no escape (e.g. the Vogel Drinking conflict test where the water sipper delivers a shock on a random schedule). In fact, the research literature involving animal models of anxiety hardly ever mention the possibility that such studies might be causing distress. This is in very marked contrast to the literature involving animal models of pain and probably stems from the fact that "anxiety" in animals is either denied altogether (e.g. Cassano, 1983) or greatly discounted as a source of distress.

Nevertheless, Gray's 1988 review of anxiety research (and his proposal that "anxiety" is part of a Behavioral Inhibition System that provides survival benefits to vertebrates) stresses the similarities between rodent behaviors in response to situations that produce anxiety and human behaviors. He notes that anxiogenic and anxiolytic drugs produce very similar outcomes in rodents and monkeys to those seen in humans.

Rodent models dominate this research so the discussion below is limited to their use. As will be seen in the representative sample of tests presented in Table 2, the vast majority of the nongenetic manipulations (Models 1-9) are based upon the strategy of creating a conflict between the natural curiosity of an animal to explore novel nonsocial or social situations and the need to avoid dangerous and aversive situations. Models 10-12 expose animals to traumatic circumstances known to have human clinical relevance. Models 13 and 14 focus on behavioral markers of anxiety, and model 15 emphasizes genetic manipulations. Information on additional models, particularly those involving genetic manipulation, can be found in Ohl (2005).

Overview

1. The variety of models and measures found in the literature indicates substantial theoretical and methodological disarray. Specifically, there has been a dearth of research which has looked at the level of distress produced by the different models, or the relative usefulness of the various approaches, and what, if any, relationship exists between the various models and the array of human clinical disorders. This is problematic from the animal welfare perspective given the wide variation in the level of induced distress present in the various models.

2. Because the function or “meaning” of the observed behavior is vague, validation has been limited to the results of pharmacological challenges. In other words, if dosing animals with medications known to have reliable anti-anxiety effects in humans results in a decrease in the measures of animal anxiety, the model is considered validated. According to Velucci (1989): (a) The animal must be sensitive to clinically effective anxiolytics in a dose-dependent manner, (b) the relative potencies of different agents should be similar to those seen clinically in human patients, (c) the tests should distinguish the effects of anxiolytic from non-anxiolytic drugs. While these relationships are important, the fact is that many anxiolytic medications are approved for a wide range of depressive and anxiety disorders; therefore, the specifications of this approach to validation is quite limited.

3. Given that many of these models extract such a high cost to animals, the researchers need to provide a high level of justification, as would be exemplified by responses to the following kind of questions:

a. Is the understanding of the human clinical entity sufficient to warrant the development or use of an animal model in the first place?
b. What are the relative scientific merits and welfare implications of studying animals genetically predisposed to anxiety as compared to those that are not?
c. In a given experiment, which of the specific anxiety disorders is being modeled?
d. What is the empirical and theoretical relationship of the human disorder of interest to the proposed animal model? Is there evidence that data from the model under study has advanced understanding of the condition’s etiology, physiological mechanisms, and/or influenced clinical practice?
e. Given that the models range from relatively innocuous procedures like introductions into an open field to those involving shock, predators, and suffocation, does the proposed model represent the least distressful consistent with the experimental goals?
f. Does the research team contain individuals with sufficient human clinical training and experience sufficient to provide meaningful design and interpretive input?

Animal models of psychological disorders are particularly challenging in regards to animal welfare since such models often inherently cause anxiety, depression, and other states that can lead to suffering. There are a number of manipulations used to induce certain states, a total of 15 are described here for the study of anxiety in rodents alone; some causing more distress than others. While strong justification for animal use should be the first priority when assessing proposed animal use, there are a number of additional important questions to ask, including how the costs to the animal can be minimized. It is hoped that the list of important questions provided here will provide a start but that those working in the field of psychology will continue to refine manipulations and decrease distress caused when animals are actually used.

Refinement In Toxicology Testing: A Workshop to Promote Current Advances and Disseminate Best Practices

Hosted by The Humane Society of the United States

Andrew N. Rowan, Martin L. Stephens, and Kathleen M. Conlee

Techniques that refine the use of animals for research that is painful and distressful are currently being developed and used in many research facilities. Often, however, this ‘in-house’ knowledge is not published in the technical literature nor widely disseminated throughout the scientific community. The Humane Society of the United States’ (HSUS) *Pain and Distress Campaign* is committed to promoting the principles of refinement and best practices by hosting

special topic workshops and disseminating information from them. The aim is not only to further animal welfare and science, but also to initiate open dialogue with and amongst scientists on ways of eliminating pain and distress in laboratory animals. This section of Chapter 10 provides an overview of the presentations given at a workshop hosted by The HSUS in New Orleans, Louisiana on March 14, 1999 on the subject of refinement in toxicological methods.

The *'Refinement in Toxicology Testing'* workshop aimed to identify and produce guidance for the consistent use of humane endpoints as well as additional refinements, such as dosing guidelines, telemetry, and Xenogen's imaging technique. The broad application of such refinements will serve to reduce and/or eliminate the pain and distress that animals might experience during experimental procedures. The workshop presentations focused on four areas of refinements in toxicology testing: (1) empirical data to determine optimal dosing volume, (2) the frequency and routes of administration for common toxicological procedures; (3) the adverse effects of toxic chemicals, and ways to limit suffering (e.g., identifying early indicators to set humane endpoints), and (4) behavioral/clinical assessment prior to distress and/or decline to a moribund condition that may indicate timing for implementation of humane endpoints. This workshop on sought to assess current knowledge and to summarize future areas of research.

Chronic Toxicity

Chronic toxicity studies can require that animal subjects be exposed to varying doses of test agents over an extended period of time. There is an increase in the incidence of health problems over time, due to both aging and the toxicity of the test agent, raising concerns about the welfare of the animals.

Fentener van Vlissingen (The Netherlands) assessed various clinical signs and other criteria in 507 rats exposed to a test compound in a two-year carcinogenicity study. These included clinical signs (both specific and non-specific), body weight, development of masses and postmortem pathology. An overall measure of "related discomfort" was determined retrospectively, based on both clinical and pathological observations. It was concluded that humane endpoints should include both specific (e.g. anemia) and non-specific (e.g. poor general appearance) criteria. For example, animals categorized as exhibiting "serious discomfort," were found to have significant decreases or increases in body weight. It was also found that many of the animals categorized as exhibiting "serious discomfort" had only non-specific clinical signs.

The full text of Dr. Vlissingen's paper "Retrospective evaluation of clinical signs, pathology and related discomfort in chronic studies," is at http://www.hsus.org/animals_in_research/animal_testing/workshop_on_refinements_in_toxicology_testing/ (click on the section entitled "Chronic toxicity").

Non-Invasive Monitoring of Animal Pain and Distress

Telemetry provides an opportunity for remote and intensive monitoring of an animal's physiological states that provides both insights into an animal's level of pain and distress, and also generates useful data highlighting and complementing other experimental results. Telemetry devices allow precise monitoring of heart rate, blood pressure, blood flow, body temperature, intraocular pressure, and other physiological variables (e.g., Lefcourt, Erez, Varner, Barfield, and Tasch, 1999; Dinslage, McLaren, and Brubaker, 1998). Radio-telemetry techniques can be applied in all commonly used laboratory animal species, from mice to monkeys (Kramer, 2000).

The initial implantation of telemetry transmitters requires surgery and is, therefore, invasive. It has been reported that implants of an appropriate size are well tolerated by the animals (Moran, et al., 1998) but there will inevitably be acute adverse sequelae that should be allowed to resolve prior to experimental treatment. However, after the implantation of the telemetric devices, the method is non-invasive. Animals can move freely and any increase or decrease in body temperature, heart rate, and blood pressure, etc., can be measured without any handling or manipulation of the animal, thus reducing the animal's stress and the time needed for lab technicians to gather data. Animals who appear during this intensive monitoring to be physiologically compromised from the experimental procedures, i.e., their blood pressure and heart rate indicate severe distress, can be euthanized. Once removed, the telemetric devices may be used again for other animals.

In his paper presented at The HSUS workshop, Doerning (Procter & Gamble) described using telemetry to monitor the impact of routine husbandry and experimental procedures on rats. The results indicated that cage cleaning, placement into clean cages, replacement of cages on the rack, and exit of a technician from the room, produced an elevation in a range of common physiological parameters for over two hours. Figure 1 shows the impact of cage changing on various variables. Animals were divided into two groups of 7 and placed in separate racks designated Rack 1 and Rack 2. At 8:30 am, well into the rats' quiet phase, animal cages were removed from the rack; animals were then placed in clean cages and returned to rack. The first arrow on the temperature graph indicates entrance into the room and is designated as 0 hour. The second arrow indicates when the procedure was finished and the technician left the room. The data is very similar between the two groups, as is the length of time it takes each parameter to return to basal levels. It can be seen that the effect of a routine cage change elevates the physiologic parameters measured above the normal quiet phase levels for well over two hours.

The effect of routine examinations was slightly less in the rats' home cage environment than when they were in a novel environment. (Heart rates returned to normal after 45 minutes for animals examined in their home environment versus approximately 90 minutes for rats examined in the strange environment.) Similar results were found in related experiments examining changes in physiological parameters during injectable anesthesia versus injectable euthanasia, blood collection versus minor surgery, and exsanguination. (See also, Hubrecht, R., ed. (1994), A Report by the Toxicology and Welfare Working Group: Housing husbandry and welfare provision for animals used in toxicology studies, on the UFAW website at http://www.ufaw.org.uk/toxicology.php.)

The full text of Dr. Doerning's paper "Effects of Routine Animal Husbandry and Experimental Procedures on Physiological Parameters of Rats," is at www.hsus.org/animals_in_research/animal_testing/workshop_on_refinements_in_toxicology_testing (click on the section entitled "Non-invasive monitoring of animal pain and distress").

Other Non-Invasive Monitoring Methods

There are a number of new methods that reduce the amount of time animals must spend in experiments and that permit an earlier endpoint, reducing the level of distress experienced by the animal.

Contag (Xenogen) described a technique that uses *in vivo* imaging to monitor biological activity such as gene expression. Biological tissue is tagged with photoproteins (e.g. luciferase or green fluorescent protein) and the photons can be detected even when they are coming from inside the animal. For example, bioluminescent tags can be attached to infectious agents and the rate of growth and spread of the infectious agent can be easily tracked. Contag provided examples of infectious disease research that could be concluded within eight hours and long before the

animal began to display any clinical signs. Onee advantage of using bioluminescence is that each animal serves as its own control and data from the same animal can be compared from one time point to the next, thereby reducing animal numbers and animal-to-animal variation.

The full text of Contag's paper "Refinement of Animal Models by Noninvasive Monitoring of Infection and Gene Expression," is at www.hsus.org/animals_in_research/animal_testing/workshop_on_refinements_in_toxicology_testing (click on the section entitled "Other non-invasive monitoring methods").

Acute Toxicity Studies: The Search for Refinement Endpoints

Schlede (Germany) discussed the issue of humane endpoints as a method of refinement in studies of acute oral toxicity, skin and eye irritation and corrosion, and skin sensitization. She examined the results of numerous tests performed in various laboratories and reported that there was a large variation in clinical signs documented by laboratories, as well as an enormous number of combinations of clinical signs observed. Clinical signs observed in animals that eventually died during the testing were examined to determine if they were "alert signs" for impending death. For example, in acute oral toxicity testing, convulsions, lateral recumbency and tremors were found to be 'alert signs' for the impending death of rats. However, Schlede reported that there was no obvious single sign that could be reliably used to alert the toxicologist to proceed with euthanasia.

The full text of Schlede's paper "Humane endpoints in toxicity testing," is at www.hsus.org/animals_in_research/animal_testing/workshop_on_refinements_in_toxicology_testing (click on the section entitled "Acute toxicity studies: the search for refinement endpoints").

Dosing Data and Volume

Smith (United Kingdom) introduced a draft 'best practice' guide on the administration of substances in preclinical toxicology studies. Dr. Smith presented details of the guide, including the guide's objectives, factors affecting the development of the guide, useful tables of best practice administration volumes and routes for common laboratory species, as well as a "decision tree" (see Figure 2) for selecting the optimal vehicle of delivery.

Smith emphasized the correlations between refinement, validity of scientific data and ethical concerns. An example of a refinement technique is his recommendation that small-scale pilot studies be carried out on any new formulation before committing to larger-scale studies.

Richmond (United Kingdom) presented a summary of findings from the British Home Office on elements of standardizing procedures and identifying best practices in toxicology testing within Britain. He offered refinements in terms of recommended limits on dosing volumes per species, frequency and route. Table 3 lists Home Office guidelines regarding limit volumes for administration of substances. However, this guide is in the process of being revised following the draft guide discussed by Smith.

The full texts of the above two papers entitled "Dosing limit volumes: A European View' and "Dosing limit volumes: The United Kingdom View-Past and Present," is at www.hsus.org/animals_in_research/animal_testing/workshop_on_refinements_in_toxicology_testing (click on the section entitled "Dosing data and volume").

While not all areas regarding the use of animals in toxicity studies are addressed here, the information from this workshop provided some general principles that should be considered and applied, regardless of the specific use of animals. For example, the way in which a substance is administered contributes to the suffering that the animal may experience; therefore the procedure used should be carefully considered and continually refined. How the animals are observed, monitored and how information regarding their welfare is collected, such as the use of telemetry described here, is also critically important in terms of minimizing pain and distress and establishing humane endpoints.

Finally, a recent report by the National Academy of Science emphasized a vision for the future of toxicity testing that would not include the use of animals and would instead use new methods in computational biology and a comprehensive array of in vitro tests based on human biology. Given how rapidly the field of toxicology is developing, those using animals for this purpose are encouraged to keep abreast of new developments that not only refine the use of animals, but replace them altogether.

References

Anon (1967) *Humane Killing of Animals*, UFAW, Herts, UK

Anton,F., Euchner,I. & Handwerker,H.O. (1992) Psychophysical examination of pain induced by defined CO_2 pulses applied to the nasal mucosal. *Pain* 49, 53-60.

AVMA (2000) Report of the AVMA panel on euthanasia. *Journal of the American Veterinary Medical Association* 218, 669-696.

Bar-Yosef, S., Melamed, R. ,Page, G.G. , Shakhar, G. , Shakhar, K. , Ben-Eliyahu, S. (2001) Attenuation of the tumor-promoting effect of surgery by spinal blockade in rats. *Anesthesiology* 94:1066-73.

Beilin, B., Shavit, Y. , Trabekin, E., Mordasher, B., Mayburd, E. , Zeidel, A., Bessler, H. (2003) The effects of postoperative pain management on the immune response to surgery. *Anesthesia and Analgesia* 97, 822-7.

Ben-Eliyahu, S. , Page, G.G. , Yirmiya, R. , Shakhar, G. (1999) Evidence that stress and surgical interventions promote tumor development by suppressing natural killer cell activity. *International Journal of Cancer* 80, 880-88.

Blackmore,D.K. 1993. Euthanasia; not always eu. *Australian Veterinary Journal* 10, 409-413.

Blackshaw,J.K., Fenwick,D.C., Beattie,A.W. & Allan, D.J. (1988) The behaviour of chickens, mice and rats during euthanasia with chloroform, carbon dioxide and ether. *Laboratory Animals* 22, 67-75.

Bowlby, J. (1988). *A secure base: Parent-child attachment and healthy human development.* New York: Basic Books.

Cassano, G.B. (1983). What is pathological anxiety and what is not? In The *Benzodiazepines: from Molecular Biology to Clinical Practice*, ed. E.Costa, pp 287-293. New York, Raven Press.

Coenen, A.M.L., Drinkenburg,W.H.I.M., Hoenderken,R. & van Luijtelaar,E.L.J.M. (1995). Carbon dioxide euthanasia in rats; oxygen supplementation minimizes signs of agitation and asphyxia. *Laboratory Animals* 29, 262-268.

Contag, C.H., Contag, P.R., Mullins, J.I., Spilman, S.D., Stevenson, D.K., and Benaron, D.A. (1995) Photonic detection of bacterial pathogens in living hosts. *Molecular Microbiology*, 18(4): 593-603.

Contag, P.R. (2008) Bioluminescence imaging to evaluate infections and host response in vivo. *Methods in Molecular Biology*, 415:101-118.

Danneman, P.J., Stein, S. & Walshaw, S.O. (1997). Humane and practical implications of using carbon dioxide mixed with oxygen for anaesthesia or euthanasia or rats. *Laboratory Animal Science* 47, 376-384.

Datta V., Bann S., Beard J., Mandalia M., Darzi A. (2004) Comparison of bench test evaluations of surgical skill with live operating performance assessments. *Journal of the American College of Surgery* 199, 603-606.

Davis, J. (1999/2000) The triple A approach to ensuring welfare. AWIC Bulletin, 19 (3-4). Retrieved September 2, 2009 from http://www.nal.usda.gov/awic/newsletters/v10n3/10n3davi.htm

Desborough J.P. (2000) The stress response to trauma and surgery. *British Journal of Anaesthesia,* 85, 109-17.

Diehl, K.H., Hull, R., Morton, D.B., Pfister, R., Rabemampianina, Y., Smith, D., Vidal, J.M. and van de Vorstenbosch, C. (2001) A good practice guide to the administration of substances and removal of blood, including routes and volumes. *Journal of Applied Toxicology*, 21, 15-23.

Dinslage, S., McLaren, J., and Brubaker, R. (1998). Intraocular pressure in rabbits by telemetry II: effects of animal handling and drugs. *Investigative Ophthalmology and Visual Sci*ence, 39(12): 2485-9.

Eriksson, K. and Holmgren, J. (2002) Recent advances in mucosal vaccines and adjuvants, *Current Opinion in Immunology*, 14 (5): 666-672.

EU Working Party (1996) Recommendations for euthanasia of experimental animals: Part 1. *Laboratory Animals* 30, 292-316.

EU Working Party (1997) Recommendations for euthanasia of experimental animals: Part 2. *Laboratory Animals* 30, 1-32.

Ewbank,R. (1983) Is CO_2 euthanasia humane? *Nature* 305, 268.

Fenwick,D.C. & Blackshaw,J.K. (1989) Carbon dioxide as a short-term restraint anaesthetic in rats with sub-clinical respiratory disease. *Laboratory Animals* 23, 220-228.

Foss, D.L. and Murtaugh, M.P. (2000) Mechanisms of vaccine adjuvanticity at mucosal surfaces. *Animal Health Research Reviews*, 1(1): 3-24.

Georgeson, K.E. and Owings, E. (2000) Advances in minimally invasive surgery in children. *The American Journal of Surgery* 180: 362-364.

Gregory,N.G., Mohan-Raj,A.B. & Audsley,A.R.S. (1990) Effect of CO_2 on man. *Fleischwirtschaft* 70, 1173-1174.

Gregory, N.G. (1998) Physiological mechanisms causing sickness behaviour and suffering in diseased animals. *Animal Welfare* 7:293-305.

Gregory, N. G. (2004) *Physiology and Behaviour of Animal Suffering.* Universities Federation for Animal Welfare. Blackwell Science: Oxford.

Hackbarth,H., Küpper,N. & Bohnet,W. (2000) Euthanasia of rats with carbon dioxide - animal welfare aspects. *Laboratory Animals* 34, 91-96.

Hamm, T.E. (1995) Proposed Institutional Animal Care and Use Committee Guidelines for Death as an End Point in Rodent Studies, *Contemporary Topics in Laboratory Animal Science*, 34, 69-71.

Harlow, H.F., Harlow, M.K., Suomi, S.J. (1971). From thought to therapy: Lessons from a primate laboratory. *American Scientist*, 59, 538-549.

Hau, J. and Hendriksen, C.F.M. (2005) Refinement of polyclonal antibody production by combining oral immunization of chickens with harvest of antibodies from the egg yolk. *ILAR Journal*, 46, 294-299.

Hewett,T.A., Kovacs,M.S., Antwohl,J.E. & Taylor-Bennett,B. (1993) A comparison of euthanasia methods in rats, using carbon dioxide in pre-filled and fixed flow rate filled chambers. *Laboratory Animal Science* 43, 573-582.

Home Office (1997) *The Humane Killing of Animals under Schedule 1 to the Animals (Scientific Procedures) Act 1986.* HMSO, London.

Hubrecht, R., ed. (1994). A Report by the Toxicology and Welfare Working Group: Housing husbandry and welfare provision for animals used in toxicology studies: Results of a UK questionnaire on current practice. UFAW Web site, http://www.users.dircon.co.uk/~ufaw3/toxintro.htm

ILAR Journal (2005) *Immunization procedures and adjuvant products*. 46: 227-319.

Iwanaka, T., Arkovitz, M.S., Arya, G., Ziegler, M.M. (1997) Evaluation of operative stress and peritoneal macrophage function in minimally invasive operations. *Journal of the American College of Surgeons,* 184:357-63.

Iwarsson,K. & Rehbinder,C. (1993) A study of different euthanasia techniques in guinea pigs, rats and mice. Animal response and post-mortem findings. *Scandinavian Journal of Laboratory Animal Science* 4, 191-205.

Kahn, M.S., Bann, S.D., Darzi, A., Butler, P.E. (2003) Assessing surgical skill. *Plastic and Reconstructive Surgery* 112, 1886-1889.

Karas AZ, Gostyla K, Aronovits M, Karas RH. (2001) Diminished Body Weight and Activity Patterns in Mice Following Surgery: Implications for Control of Postprocedural Pain/Distress in Laboratory Animals, *Contemporary Topics in Laboratory Animal Science*, 40,83.

Kehlet, H. (2000) Manipulation of the metabolic response in clinical practice. *World Journal of Surgery*. 24:690-95.

Kelley, K. W. and Dantzer, R. (2007) Twenty years of research on cytokine-induced sickness behavior. *Brain, Behavior, and Immunity* 21:153-160.

Kessler, R. C., McGonagle, K. A., Zhao, S., Nelson, C. B., Hughes, M., Eshleman, S., Wittchen, H. U., & Kendler, K. S. (1994). Lifetime and 12-month prevalence of DSM-III-R psychiatric disorders in the United States. Results from the National Comorbidity Survey. *Archives of General Psychiatry,* 51, 8–19.

Kiecolt-Glaser, J.K., Page, G.G., Marucha, P.T., MacCallum, R.C., Glaser, R. (1998) Psychological influences on surgical recovery: perspectives from psychoneuroimmunology. *American Psychologist*. 53, 1209-18.

Kohler,I., Meier,R., Busato,A., Neiger-Aeschbacher,G. & Schatzmann,U. (1999) Is carbon dioxide ($CO_{2)}$ a useful short acting anaesthetic for small laboratory animals? *Laboratory Animals* 33, 155-161.

Komai,M. & Bryant,B.P. (1993) Acetazolamide specifically inhibits lingual trigeminal nerve responses to carbon dioxide. *Brain Research* 612, 122-129.

Kort WJ, Hekking-Weijma JM, TenKate MT, Sorm V, van Strik R. (1998) A Microchip Implant System as a Method to Determine Body Temperature of Terminally-ill rats and Mice, *Laboratory Animals*, 32, 260-269.

Kramer, K. (2000) *Applications and Evaluation of Radio-Telemetry in Small Laboratory Animals*. The
Netherlands.

Krarup A, Chattopadhyay P, Bhattacharjee A, Burge JR, Ruble GR. (1999) Evaluation of Surrogate Markers of Impending Death in the Galactosamine-Sensitized Murine Model of Bacterial Endotoxemia, *Laboratory Animal Science*, 49:5, 545-550.

Lambooij, E., Gerritzen, M.A., Engel,B., Hillebrand, S.J.W., Lankhaar, J. & Pieterse, C. (1999) Behavioural responses during exposure of broiler chickens to different gas mixtures. *Applied Animal Behaviour Science* 62, 255-265.

Leach, M.C., Bowell, V.A., Allan,T.F. & Morton, D.B. (2002a) The aversion to various concentrations of different inhalational general anaesthetics in rats and mice. *The Veterinary Record*, 50(26): 808-815.

Leach, M.C., Bowell,V.A., Allan,T.F. & Morton,D.B. (2002b) Aversion to gaseous euthanasia agents in rats and mice. *Comparative Medicine* 52, 249-257.

Leach M.C., Bowell, V.A., Allan, T. & Morton, D.B. (2003). Measurement of aversion to determine humane methods of anaesthesia and killing. *Animal Welfare*, 13, S77-S86.

Lefcourt, A.M., Erez, B., Varner, M.A., Barfield, R., and Tasch, U. (1999). A noninvasive radiotelemetry system to monitor heart rate for assessing stress responses of bovines. *Journal of Dairy Science,* 82(6):1179-87.

Lucke, J.N. (1979) Euthanasia in small animals. *The Veterinary Record* 104, 316-318.

Ludders,J.W., Schmidt,R.H., Dein,F.J. & Klein,P.N. (1999) Drowning is not euthanasia. *Wildlife Society Bulletin* 27, 666-670.

MacArthur,J.A. (1978) Carbon dioxide euthanasia of small animals (including cats). In: *Humane Killing of Animals,* London, Universities Federation for Animal Welfare.

Maier, S.F. and Watkins, L.R. 1998. Cytokines for psychologists: implications of bidirectional immune-to-brain communications for understanding behavior. *Psychological Review* 105:83-107.

Mason G, Wilson D, Hampton C, Wurbel H. (2004) Non-invasively Assessing Disturbance and Stress in Laboratory Rats by Scoring Chromodacryorrhoea, *Alternatives to Laboratory Animals*, 32:Suppl. 1, 153-159.

Medina L.V. (2004) How to Balance Humane Endpoints, Scientific Data Collection, and Appropriate Veterinary Interventions in Animal Studies, *Contemporary Topics in Laboratory Animal Science*, 43, 56-62.

Moran, M.M., et al (1998) Size constraints of telemeters in rats. *Journal of Applied Physiology*, 85(4): 1564-1571.

Morton DB. (2000) A Systematic Approach for Establishing Humane Endpoints*, ILAR Journal*, 41, 80-86.

Morton DB, Hawkins P, Bevan R, Heath K, Kikrwood J, Pearce P, Scott E, Whelan G, Webb A. (2003) Refinements in Telemetry Procedures, *Laboratory Animals*, 37, 261-299.

Mouton,W.G., Naef,M., Bessell,J.R., Otten,K.T., Wagner,H.E. & Maddern,G.J. (2001) A randomized controlled trial to determine the effect of humidified carbon dioxide (CO2) insufflation on postoperative pain following thoracoscopic procedures. *Surgical Endoscopy-Ultrasound and Interventional Techniques* 15, 579-581.

Mullenax, C. H. & Dougherty, R. W. (1963) Physiologic responses of swine to high concentrations of inhaled carbon dioxide. *American Journal of Veterinary Research*, 24, 329-332.

Ohl, F. (2005) Animal models of anxiety. *Handbook of Experimental Pharmacology*, 169:35-69.

Olfert E.D. and Godson D.L. (2000) Humane Endpoints for Infectious Disease Animal Models, *ILAR Journal*, 41, 99-104.

Paton,W. (1983) Is CO_2 euthanasia humane? *Nature* 305, 268.

Pavlov, I.P. (1928). *Lectures on conditioned reflexes: The higher nervous activity of animals*. London: Lawerence and Wishart

Peppel,P. & Anton,F. (1993) Responses of rat medullary dorsal horn neurons following intranasal noxious chemical stimulation: effects of the stimulus intensity, duration, and interstimulus interval. *Journal of Neurophysiology* 70, 2060-2275.

Perkins, F.M and Kehlet, H. (2000) Chronic pain as an outcome of surgery; a review of predictive factors. *Anesthesiology* 93:1123-33.

Public Health Service (2002) Policy on the Humane Care and Use of Laboratory Animals.

Raj, A.B.M. & Gregory,N.G. (1994) An evaluation of humane gas stunning methods for turkeys. *The Veterinary Record* 135, 222-223.

Raj, A.B.M. & Gregory,N.G. (1995) Welfare implications of the gas stunning of pigs: Determination of aversion to the initial inhalation of carbon dioxide or argon. *Animal Welfare* 4, 273-280.

Raj, A.B.M. (1999) Behaviour of pigs exposed to mixtures of gases and the time required to stun and kill them: welfare implications. *The Veterinary Record* 144, 165-168.

Raj A.B.M, Leach M.C. & Morton D.B.M (2004) Carbon dioxide for euthanasia of laboratory animals. *Comparative Medicine* 54, 470-471.

Raj, A.B.M. & Whittington, P.E. (1995) Euthanasia of day-old chicks with carbon dioxide and argon. *The Veterinary Record* 136, 292-294.

Regier, D. A., Narrow, W. E., Rae, D. S., Manderscheid, R. W., Locke, B. Z., & Goodwin, F. K. (1993). The de facto US mental and addictive disorders service system.
Epidemiologic Catchment Area prospective 1-year prevalence rates of disorders and services. *Archives of General Psychiatry, 50*, 85–94.

Schade, R., Staak, C., Hendriksen, C., Erhard, M., Hugl, H., Koch, G., Larsson, A., Pollmann, W., van Regenmortel, M., Rijke, E., Spielmann, H., Steinbusch, H., & Straughan, D. (1996). The production of avian (egg yolk) antibodies: IgY. *Alternatives to Laboratory Animals*, 24(6): 925–933.

Seligman, M. (1975). *Helplessness*. San Francisco: Witt, Freeman &Co.

Shafer, M. (2003) Cytokines and peripheral analgesia. *Advances in experimental medicine and biology*. 521,40-50.

Sheeran, P. and Hall, G.M. (1997) Cytokines in anesthesia. *British Journal of Anesthesia* 78: 201-219.

Smith,W. & Harrap,S.B. (1997) Behavioural and cardiovascular responses of rats to euthanasia using carbon dioxide. *Laboratory Animals* 31, 337-346.

Thurauf, N., Friedel,I. & Hummel,C. (1991) The mucosal potential elicited by noxious chemical stimuli with CO_2 in rats: is it a peripheral nociceptive event? *Neuroscience Letters* 128, 297-300.

Toth LA. (2000) Defining the Moribund Condition as an Experimental Endpoint for Animal Research, *ILAR Journal*, 41, 72-79.

Turner, R.D., Held, S.D.E., Hirst, J.E., Billinghurst, G. and Wooton, R.J. (1997) An immunological assessment of group-housed rabbits. *Laboratory Animals*, 31, 362-372.

Ullman-Cullere MH and Foltz CJ. (1999) Body Condition Scoring: A Rapid and Accurate Method for Assessing Health Status in Mice, *Laboratory Animal Science*, 49, 319-323.

United States Department of Agriculture (2002) Animal Welfare Act and Animal Welfare Regulations.

van der Meer M, Rolls A, Baumans V, Olivier B, van Zutphen LFM. (2001) Use of Score Sheets for Welfare Assessment of Transgenic Mice, *Laboratory Animals*, *35(4)*, 379-389.

van Luijtelaar,G. & Coenen,A. (1999) Inhalational euthanasia in broiler chickens. *World Poultry* 15, 40-43.

Velucci, S. (1989). Anxiety. In R. Webster & C. Jordan (Eds.) *Neurotransmitters, drugs and disease.* London: Blackwell Scientific, 394-427.

Vlach KD, Boles JW, Stiles, BG. (2000) Telemetric Evaluation of Body Temperature and Physical Activity as Predictors of Mortality in a Murine Model of Staphylococcal Enterotoxic Shock, *Comparative Medicine*, 50,160-166.

Walsh, P.J., Remedios, A.M., Ferguson, J.F., Walker, D.D., Cantwell, S., Duke, T. (1999) Thoracoscopic versus open partial pericardectomy in dogs: comparison of postoperative pain and morbidity. *Veterinary Surgery* 28, 472-79.

Warn PA, Brampton MW, Sharp A, Morrissey G, Steel N, Denning DW, Priest T. (2003) Infrared Body Temperature Measurement of Mice as an Early Predictor of Death in Experimental Fungal Infections, *Laboratory Animals*, 37, 126-131.

Weise, M., Kramer, J., Bernsmann, K., Ernst Willburger, R. (2004) The related outcome and complication rate in primary lumbar microscopic disc surgery depending on the surgeon's experience: comparative studies. *Spine Journal: Official Journal of the North American Spine Society*, 4, 550-6.

Wolpe, J., Salter, A. and Reyna, L.J. (1964). *The conditioning therapies*. New York: Holt, Rinehart, and Winston for a review of these studies

Wong JP, Saravolac EG, Clement JG, Nagata LP. (1997) Development of a Murine Hypothermia Model for Study of Respiratory Tract Influenza Virus Infection, *Laboratory Animal Science*, 47, 143-147.