Comparative Medicine
Copyright 2005
by the American Association for Laboratory Animal Science

Vol 55, No 3
June 2005
Pages 275-281

Euthanasia of Neonatal Mice with Carbon Dioxide

Kathleen Pritchett, DVM,[1,*] Dorcas Corrow,[2] Jason Stockwell, PhD,[3] and Abigail Smith, PhD[4]

Exposure to carbon dioxide (CO_2) is the most prevalent method used to euthanize rodents in biomedical research. The purpose of this study was to determine the time of CO_2 exposure required to euthanize neonatal mice (0 to 10 days old). Multiple groups of mice were exposed to 100% CO_2 for time periods between 5 and 60 min. Mice were placed in room air for 10 or 20 min after CO_2 exposure, to allow for the chance of recovery. If mice recovered at one time point, a longer exposure was examined. Inbred and outbred mice were compared. Results of the study indicated that time to death varied with the age of the animals and could be as long as 50 min on the day of birth and differed between inbred and outbred mice. Institutions euthanizing neonatal mice with CO_2 may wish to adjust their CO_2 exposure time periods according the age of the mice and their genetic background.

Mice and rats are the most commonly used mammals in today's biomedical research programs. The number of mice and rats used in nonfederal research facilities in the United States in 1998 was estimated to be 20 million (18). Since that time, the numbers used yearly have risen as the usefulness of genetically manipulated rodents has continued to grow and biomedical research programs have expanded. Because euthanasia is a crucial and sensitive part of laboratory mouse husbandry, it should be performed quickly and painlessly (for the mouse), efficiently and aesthetically (for the worker), and cost-effectively (for the institution).

Recommendations on euthanasia procedures for laboratory animals have been proposed by many authors. The American Veterinary Medical Association (AVMA) has convened a Panel on Euthanasia (the Panel) in 1972, 1978, 1986, 1993, and 2000 to address the humaneness of euthanasia methods currently in use and to make recommendations for euthanasia of different species. The Panel's 2000 Report briefly addresses euthanasia of neonatal animals, undoubtedly due to a lack of published literature, stating in the section on inhalant agents: "Neonatal animals appear to be resistant to hypoxia…The Panel recommends that inhalant agents not be used alone in animals less than 16 weeks old except to induce loss of consciousness, followed by the use of some other method to kill the animal." (2) This recommendation by the Panel was made after reviewing the work of Glass and colleagues, who evaluated the euthanasia of newborn dogs, rabbits, and guinea pigs with nitrogen gas, but did not examine other laboratory rodent species (10). This recommendation fails to take into account that many laboratory rodents are fully mature at 16 weeks, and has the potential to limit researchers and animal producers greatly. The AVMA Panel document further addresses the use of inhalant agents, specifically carbon dioxide (CO_2) in neonates as follows: "Thus, CO_2 concentrations for newly hatched chickens and neonates of other species should be especially high. A CO_2 concentration of 60% to 70% with a 5-min exposure time appears to be optimal" (2). The citation that follows this statement is one that discusses CO_2 euthanasia of newly hatched chickens (13).

The present study does not intend to evaluate the merits of the use of CO_2 to euthanize laboratory animals but rather to elucidate its proper use in the euthanasia of neonatal mice. Our study has expanded upon and clarified the results of Avery and Johlin, Reiss and Haurowitz, and Klaunberg and colleagues (1, 16, 24) by using microbiologically and genetically defined animals tested at exact time points. In the present study, the aim was to determine the length of exposure to 100% CO_2 necessary to euthanize 100% of mice 0 to 10 days of age.

Materials and Methods

Animals. Pregnant Crl:CD1(ICR), C57BL/6NCrl, FVB/NCrl, and BALB/cAnNCrl female mice were obtained from Charles River Laboratories (CRL; Wilmington, Mass.) and pregnant A/J, CBA/J, C57BL/6J, BALB/cJ, BALB/cByJ, C3H/HeJ, C3HeB/FeJ, SJL/J, and DBA/2J female mice were obtained from The Jackson Laboratory (TJL; Bar Harbor, Maine). Studies were conducted jointly between the two institutions. Experiments at CRL were conducted at CRL's Wilmington, Mass., facility, with approval from their institutional animal care and use committee (IACUC). Experiments with animals from TJL were conducted at TJL's Bar Harbor, Maine, facility, with approval from the TJL IACUC. All work was conducted according to standards set forth in the *Guide for the Care and Use of Laboratory Animals*.

Animals were housed in solid-bottomed cages, with contact bedding of either pine shavings (Crobb Box Co., Ellsworth, Maine) or a paper-based product (CareFresh, Midwest Filtration, Cincinnati, Ohio). Cages were changed once weekly. A minimum of 15 air changes per hour was maintained in animal holding rooms. Animals were kept on a 12:12 or 14:10-h light:dark cycle and provided ad libitum access to sterile or acidified water and sterile feed (Lab Diet 5K52 or 5L79, Purina Mills, Richmond, Ind.). All animals were from colonies that tested negative for

Received: 1/14/05. Revision requested: 2/26/05. Accepted: 2/28/05.
[1]Animal Services, Charles River Laboratories, 251 Ballardvale St, Wilmington, Massachusetts 01887; [2]Laboratory Animal Health Services, The Jackson Laboratory, 600 Main St., Bar Harbor, Maine 04609; [3]United States Geological Survey—Great Lakes Science Center, Lake Superior Biological Station, 2800 Lakeshore Dr., Ashland, Wisconsin 54806; [4]University Laboratory Resources and Department of Pathobiology, School of Veterinary Medicine, University of Pennsylvania, 3800 Spruce Street, Suite 177, Philadelphia, Pennsylvania 19104.
[*]*Corresponding author.*

Sendai virus, pneumonia virus of mice, mouse hepatitis virus, minute virus of mice, mouse parvovirus, Theiler's mouse encephalomyelitis virus, reovirus type 3, mouse adenovirus, polyoma virus, K virus, mouse cytomegalovirus, rotavirus (epizootic diarrhea of infant mice virus), mouse thymic virus, lymphocytic choriomeningitis virus, hantavirus, lactate dehydrogenase elevating virus, ectromelia, *Mycoplasma pulmonis*, *Helicobacter* spp., *Salmonella* spp., *Pasteurella* spp. (including *P. multocida* and *P. pneumotropica*), *Corynebacterium kutscheri*, *Citrobacter rodentium*, and *Streptobacillus moniliformis*. Animals were also free of endo- and ectoparasites. Pregnant females were allowed to litter naturally, and day of birth was recorded as day 0 of the animal's life. Preweanlings used for experiments were removed arbitrarily from females at a particular day of age and combined into experimental groups. Two broad age groups were evaluated; all animals 0 to 10 days of age are defined as preweanlings, and adults are animals older than 6 weeks.

Procedures. Three pilot studies were performed: one in which same-sex groups of male and female preweanlings were compared, one in which groups of mice from different inbred strains were compared, and one in which chambers precharged with CO_2 were compared with chambers empty of gas. No differences were observed between groups in any pilot experiment, so mice of the same age were combined without regard to sex, and inbred animals were combined without regard to strain. No differences were found in times to death in animals in precharged or uncharged chambers.

No fewer than five but no more than 10 mice of the same age comprised each experimental group. Groups were placed in a clear gusseted plastic bag of 17 × 7.5 × 39 cm (low-density polyethylene, 6 × 3 × 15 in., Polymer Packaging Inc, North Canton, Ohio) containing a small amount of room air. The bag was filled to 4 liters in volume with 100% CO_2 delivered at a rate of 0.5 liter/sec. CO_2 exposure time was measured from the introduction of gas into the bag. The filled bag was sealed by either tying a knot in the bag or by twisting the top and sealing the twisted portion with a binder clip. The filled bag was placed in a bedded cage for the duration of the exposure. All animals had sufficient room to stand on the bottom of the bag. To reduce animal numbers used in this experiment, a pilot exposure at 0 days of age was performed with limited numbers (10 per time point) of animals, and 60 min was chosen for the upper limit of CO_2 exposure, based on this pilot experiment. We chose 5 min as the lower limit of preweanling exposure, according to the AVMA Panel's recommendation and policies at many institutions requiring a 5-min exposure to CO_2. Animals were exposed to CO_2 for 5 to 60 min for preweanlings and 30 or 60 sec for adults. Animals were observed for the first 30 sec after CO_2 exposure and then at 1-min intervals until 5 min. After 5 min, animals were monitored at 5-min intervals until the end of the exposure. At the end of the CO_2 exposure period, animals were removed from the plastic bag, placed in a bedded cage, and allowed to recover. Animals were monitored for recovery for either 20 min (days 0 to 5) or 10 min (days 6 to 10 or adult). Animals were considered to have recovered if they took one breath during the recovery period. If an animal from a group recovered, the CO_2 exposure time was lengthened by 5 min for the next group of that age. The criterion for selecting a time point at which successful euthanasia was achieved was that time point at which 100% of the animals succumbed. Animals were not palpated or instrumented to detect cessation of heartbeat; failure to recover when exposed to room air was considered death. Mice that recovered were euthanized using a physical method (decapitation or cervical dislocation).

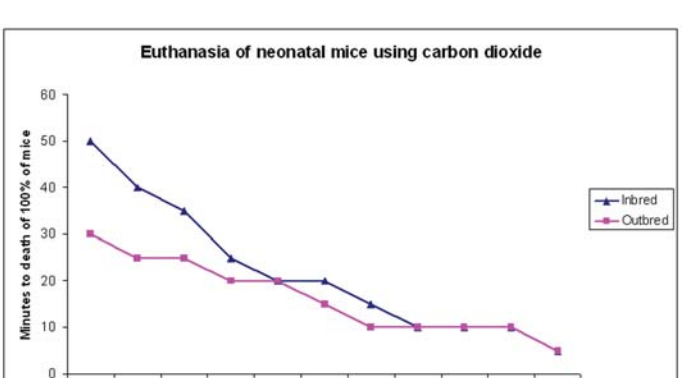

Figure 1. CO_2 exposure time (min) required to produce death of 100% of the inbred and outbred mice tested.

Unconsciousness was determined by loss of righting reflex, cessation of spontaneous respiration, and failure to respond to nocioceptive stimuli (e.g., toe or tail pinch). The mice were not palpated for a heartbeat during the recovery period, as all direct stimulation of the animals was avoided. For example, when the mice were placed in the bedded cage to recover from the CO_2 exposure, they were arranged so they did not touch each other. If an animal did recover, its respiratory efforts were not allowed to stimulate a neighbor. Mice were left at room temperature to recover to simulate conditions in which animals might recover from euthanasia. All mice were confirmed to be dead before being placed in a carcass freezer (a normal method at both CRL and TJL of holding euthanized animals before final disposition).

This study was designed to make recommendations for the euthanasia of neonatal mice with CO_2. As such, the study used an absolute criterion, 100% mortality, as an endpoint. Although the data do not lend themselves to statistical analysis, there are several fail-safes that were introduced into the study to minimize the chance that the 100% mortality seen was an anomalous occurrence. After the pilot study, which used a limited number of animals (10 per time point) to determine approximate times around which to evaluate initial exposure, at least three separate groups of 10 neonates were exposed to CO_2 at each time point. For the time points at which mice recovered, recovery never took place solely within a single group of exposed animals. Because any recovery was considered an undesirable outcome, even if due to a failure of the euthanasia chamber, that time point would have been deemed unacceptable and the next time point tested. Once the 100% mortality time point was reached, another three groups of 10 animals were tested at the next time point to examine whether the 100% mortality found at the prior time point was an anomaly.

Results

Preweanling animals did not differ in their susceptibility to CO_2 by sex, but they did differ in their susceptibility to CO_2 by age. Outbred preweanling animals differed markedly from inbred preweanlings in time to death between days 0 and 8. Inbred preweanlings were less susceptible than outbred to the effects of CO_2 from days 0 to 8 (Fig. 1). Inbred preweanlings did not

Table 1. Numbers of neonatal and adult mice tested

		Outbred animals		Inbred animals	
Age (days)	CO_2 exposure time (min)	No. tested	No. euthanized	No. tested	No. euthanized
0	15	30	11	NT	NT
	20	30	11	10	6
	25	30	27	10	6
	30	30	30	10	5
	40	10	10	30	22
	50	10	10	30	30
	60	NT	NT	30	30
1	15	30	10	NT	NT
	20	30	28	NT	NT
	25	30	30	NT	NT
	30	30	30	30	24
	35	NT	NT	30	28
	40	NT	NT	37	37
	45	NT	NT	30	30
2	15	30	8	NT	NT
	20	30	29	NT	NT
	25	30	30	30	25
	30	30	30	30	28
	35	NT	NT	35	35
	40	NT	NT	30	30
3	15	30	26	NT	NT
	20	30	30	30	21
	25	30	30	30	30
	30	30	30	30	30
4	10	62	40	NT	NT
	15	30	28	30	22
	20	30	30	30	30
	25	NT	NT	29	29
5	5	46	0	NT	NT
	10	54	47	33	24
	15	41	41	32	31
	20	NT	NT	31	31
6	5	40	2	22	4
	10	62	62	73	62
	15	NT	NT	31	31
7	5	46	4	11	2
	10	46	46	52	52
8	5	56	56	28	15
	10	56	56	28	28
9	5	56	56	20	16
	10	56	56	31	31
10	5	106	106	65	65
19	1	NT	NT	6	6
24	1	NT	NT	6	6
28	1	NT	NT	20	20
Adult	0.5	50	48	NT	NT
	1	49	49	114	114

Inbred strains evaluated were C57BL/6NCrl, FVB/NCrl, BALB/cAnNCrl, A/J, BALB/cJ, BALB/cByJ, C3H/HeJ, C3HeB/FeJ, DBA/2J CBA/J, C57BL/6J, and SJL/J; the one outbred stock tested was Crl:CD1(ICR).
NT, not tested.

differ in time to death by strain. Table 1 gives information on the numbers of animals tested at each age and time point. The animals used in pilot studies are added to the totals shown in Table 1. Size of preweanlings did not appear to be a factor in the susceptibility to CO_2. Preweanlings that appeared smaller than their littermates ("runts") succumbed at the same time point as did their more normally sized siblings (data not shown). Animals appeared dead (cold, cyanotic, unmoving) yet still recovered when exposed to room air, even after CO_2 exposures of 30 min or longer. This recovery took place as long as 18 min after removal from CO_2 exposure in 0- to 4-day-old mice (data not shown).

The pilot experiment at 0 days of age allowed for the closer bracketing of exposure times and reduction of animal numbers. The total number of preweanling animals tested was 2355—1287 outbred animals and 1068 inbred animals. To account for the normal variability in biological systems, numbers of pregnant animals needed to produce a certain number of pups for experimentation were calculated based on average litter sizes for the strains or stocks, and then a surplus (usually 10%) was ordered. Because of the nature of the experiments and housing and handling requirements, animals born could not be returned to stock or sold. Rather than waste any "excess" animals born, they were

Table 2. Postnatal developmental events useful for estimating the age of inbred mice to day 10

Age	Appearance
0 to 24 h	Blood red, possible milk spot, pigmented mice have dark eyes
Day 1	Deep pink, milk spot visible
Day 2	Ears appear as nubs, milk spot visible, pigment in skin begins to appear
Day 3	External ear flap begins to lift from head, milk spot visible
Day 4	External ear flap fully lifted from head and perpendicular to head, skin fully pigmented, milk spot visible
Day 5	External ear flap completely vertical (as opposed to perpendicular), skin appears much thicker (milk spot begins to disappear), incisors visible as white spots under gums
Day 6	Milk spot gone or faintly visible, colored fuzz appears behind ears or on dorsal neck, incisors erupted
Day 7	Colored fuzz begins to cover pup fully (more visible in albino animals, as dark animals may appear "linty" from cage dust)
Day 10	External ear open, pup fully haired
Day 13 or 14	Eyes begin to open, eye opening is a slit

During days 1 through 6, the same developmental events occur approximately a day earlier in outbred mice than inbred mice (e.g., outbred mice have their ears perpendicular to their heads at day 2 rather than 3).

euthanized, and data were collected.

In this same experimental system, 96% of healthy 8- to 9-week-old female Crl:CD1(ICR) mice were euthanized after 30 sec of exposure to CO_2 and 100% at 1 min of exposure to CO_2 (Table 1). Of the inbred 8- to 9-week-old female mice tested, 100% were euthanized after 1 min of exposure to CO_2. Strains tested as adults included: C57BL/6J, BALB/cJ, BALB/cByJ, C3H/HeJ, C3HeB/FeJ, SJL/J, and DBA/2J. Neither outbred nor inbred adult male mice were tested. As an absolute criterion, 100% mortality at a certain time point, was used to determine success of the test; there are no standard errors or *P* values reported for the results presented.

Age is the critical factor in determining susceptibility to CO_2; therefore, developmental cues can be used to determine the age of the animal (Table 2). If the exact age of animals to be euthanized is unknown, it is important to use physical development, and not size, as an indicator of age. If there is any question about the age of the mice, err on the side of assuming the animal's immaturity and use a longer CO_2 exposure. A sample exposure chart (Table 3) is accompanied by representative photos of animals at relevant time points (Fig. 2). The divisions chosen were based on easily recognizable physical signs (e.g., hair growth) associated with age milestones in the mouse. In Table 3, a margin of error is added to each exposure time recommendation to minimize the chance of postexposure recovery. This margin of error is added to accommodate inevitable human error and mechanical failure in any recommendation and to aid in recognition of susceptibility to CO_2 by physical signs of maturity.

As is evident in Fig. 1, the amount of exposure time necessary to euthanize animals at day 0 is much greater than at day 6. We chose 60 min as the exposure time for this age range in order to minimize the possibility of human error in properly aging pups. For example, pups at days 0, 1, and 2 are very similar in appearance, but for inbred mice, the day-2 pups will succumb 15 min faster. If an error is made in aging pups, animals might be underexposed to CO_2 and inadvertently recover. Overexposure to CO_2 will not result in the recovery of the animal. Animals are grouped by easily recognizable physical characteristics, not by time to euthanasia.

Table 3. CO_2 exposure time recommended for euthanasia of mice of various ages

	CO_2 exposure time
Nonhaired pups (0–6 days of age)	60 min
Haired pups, eyes closed (7–13 days of age)	20 min
Haired pups, eyes open, preweaning (14–20 days of age)	10 min
Weanlings and adults (21 days of age and older)	5 min

The sample exposure chart presented here applies to the exposure chamber and filling parameters used in this study. The use of other chamber types and fill rates will require separate calibration. All euthanasia times recommended in this table should be validated at each facility with euthanasia equipment currently in use.

Discussion

There is a dearth of information in the literature about euthanasia of preweanling rodents. A search of the rodent euthanasia literature published within the last 20 years found only two papers (3, 16) that addressed euthanasia of rodents younger than 6 weeks of age, and one of those was concerned solely with rats. In the most recent study performed, Klaunberg and colleagues used mice of various genotypes from a colony, including "inbred, outbred, hybrid, and mutant mice" to evaluate various methods of euthanasia of fetal and neonatal mice (16). In this study, two groups of neonates, 1 to 7 days old and 8 to 14 days old, were euthanized using either CO_2 or halothane for the first group or CO_2, halothane, or barbiturate overdose for the second group. Klaunberg and colleagues found, by using 11 neonates 1 to 7 days old and 15 neonates 8 to 14 days old, that the most rapid method of euthanasia of neonatal mice was inhalation of CO_2, with time to cardiac arrest of 4:41 min in 1- to 7-day-old mice and 3:52 min in 8- to 14-day-old mice. These investigators also noted a pattern of agonal breathing (a respiratory pattern of intermittent deep breaths followed by apnea) in neonates, which occurred after cardiac arrest (16). In two cases, 1- to 7-day-old mice revived themselves, indicating that the euthanasia was not successful and suggesting that cardiac arrest was transient and reversible.

A review of older literature provided two further studies (1, 24) that discussed the euthanasia of neonatal mice. In 1932, Avery and Johlin tested nine "white mice" at 3 to 5.5 g (no ages given) in two groups, one of four mice and the other of five. The five animals tested as one group survived a 20- to 33-min exposure, but the group of four died after a 36- to 60-min exposure (1). According to the average weights of Charles River Laboratories Crl:CD1(ICR) mice, an outbred stock, the mice evaluated were older than 3 days but younger than 21 days (15, 21). In a paper by Reiss and Haurowitz from 1929, neither the type of mouse nor animal numbers are provided, but ages in days are given. Animals were tested at 1, 3, and 7 days of age by using 100% CO_2, 75% CO_2 plus 25% O_2, and 50% CO_2 plus 50% O_2. In every case, animals survived longer at a younger age, with survival times of up to 420 min for a 1-day-old mouse in an atmosphere of 50% CO_2 plus 50% O_2 (24). In these older studies, systematic evaluation of the time to death is lacking, and the studies involved small numbers of animals of indeterminate age and unknown health status. The nature of the chamber in which the animals were exposed is not specified, nor is the method of generating CO_2.

In a valuable effort to standardize procedures and recommend best practices for veterinarians, the AVMA Panel on Euthanasia addressed euthanasia of various species and ages of animals in its 2000 report. "Acceptable" methods of euthanasia for rodents

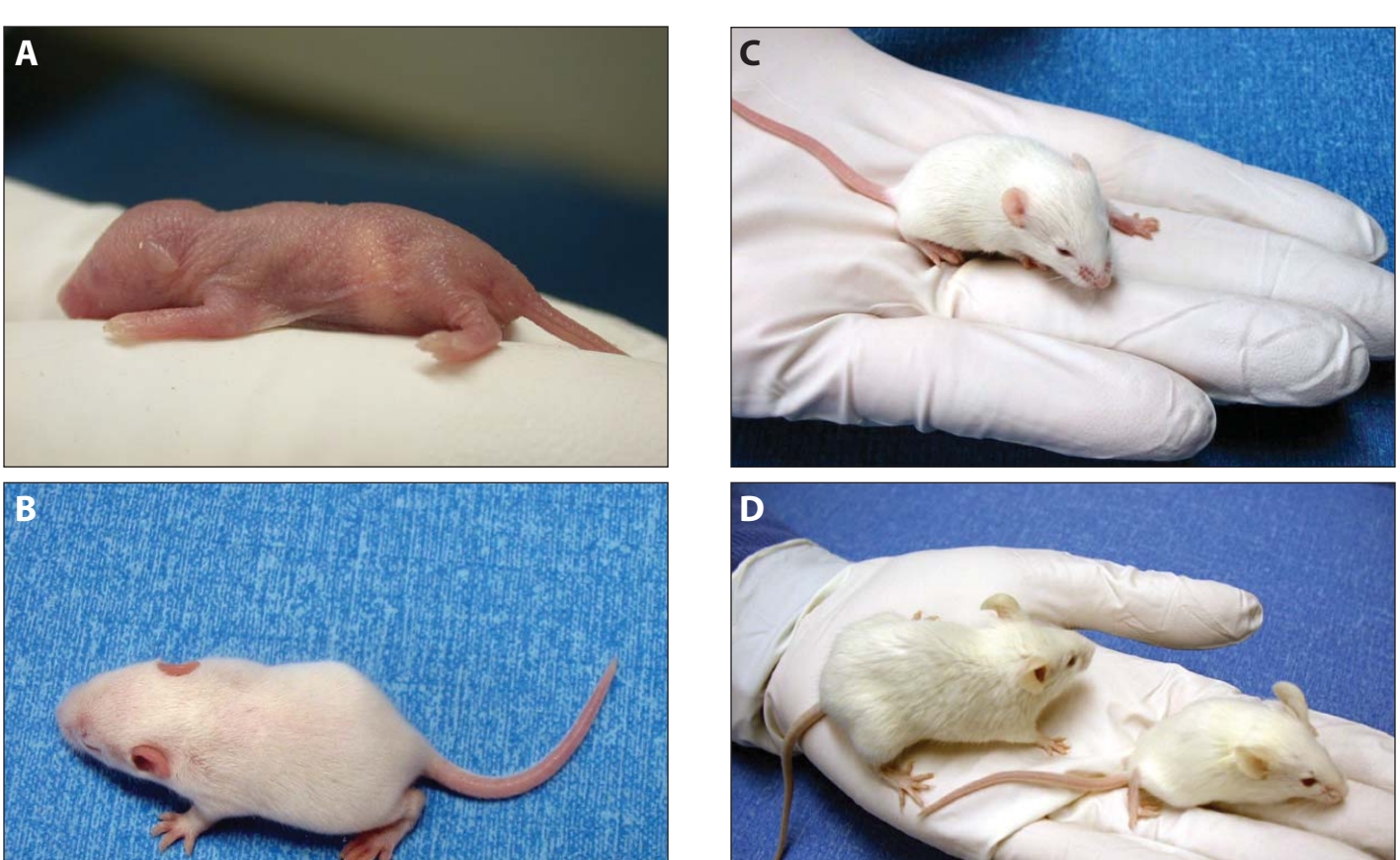

Figure 2. Representative animals at ages mentioned in Table 3. Animals are BALB/cJ mice at (A) 3 days old, (B) 10 days old, (C) 14 days old, and (D) 21 days old (right), and adult (left).

and other small mammals are: barbiturates, inhalant anesthetics, carbon monoxide, potassium chloride in conjunction with general anesthesia, microwave irradiation, and CO_2 (2). Using recommended methods of euthanasia and providing for the humane death of large numbers of neonatal mice presents several challenges. Acceptable methods for euthanasia other than CO_2 may have legally mandated record-keeping, disposal, and ordering requirements (e.g., barbiturates) or may pose occupational health hazards (e.g., carbon monoxide, inhalant anesthetics, barbiturates). Euthanasia methods other than CO_2 may also impose considerable expense (e.g. microwave irradiation), or may prove impractical for euthanizing large numbers of animals due to their labor-intensive nature (e.g. potassium chloride with general anesthesia) or their potential distress to personnel (e.g., physical methods). Methods of neonatal euthanasia used in other countries that might be practical for use on a large scale, such as immersion in liquid nitrogen, are not recommended in the United States, unless preceded by some method to render the animals insensible (2). In cases where euthanasia of large numbers of preweanling animals is necessary, institutions should consider the potential occupational health and emotional cost to personnel of the use of other methods and the relative benignity of CO_2 to both the animals and personnel. The use of CO_2 for euthanasia of adult rodents, although debated by some (12, 23), is widely accepted due to its speedy onset of effects (3, 4, 11), minimal tissue artifact production (7), and ease of use. Yet, despite CO_2's general acceptance, questions remain about ideal conditions of its use.

The AVMA Panel recommends a 5-min exposure to 60% to 70% CO_2 as sufficient for neonatal rodent euthanasia (2). Our results do not support this recommendation. In fact, we found that a 5-min exposure to CO_2 will not suffice for euthanasia of neonatal mice until at least day 10 of life. The necessity of a prolonged CO_2 exposure time for neonates can be explained when the mechanisms of action of CO_2 and innate resistance of the neonate to hypercarbia and hypoxia are examined.

Carbon dioxide is a small molecular weight molecule that quickly diffuses from the lungs into the plasma and intracellular spaces. Blood has a buffering capacity for CO_2, using the bicarbonate system to produce bicarbonate ions (HCO_3^-) to keep CO_2 levels within a tightly controlled range. When there is an excess of CO_2 in the blood, CO_2 combines with water to produce carbonic acid, which immediately dissociates into H+ and HCO_3^-. When the capacity of this buffering system is exceeded, an excess of CO_2 in the blood leads to acidosis, that is, a lowering of the pH of the blood and associated fluids. Mild respiratory acidosis leads to a compensatory increase in depth and rate of respiration in an effort to "blow off" the excess CO_2. More profound respiratory acidosis quickly suppresses the respiratory centers of the brain,

leading to a slow, gasping respiratory pattern (9, 19). The cerebrospinal fluid (CSF) lacks the buffering capacity of the blood, so the pH of CSF in acute respiratory acidosis will drop precipitously (22). This lowering of CSF pH is directly associated with anesthetic depth and insensibility to pain and results in a quick onset of stupor and coma in humans (17), in addition to the respiratory effects noted earlier. Another mechanism of action of CO_2 is the acidosis-induced depression of myocardial contractility and the direct action of CO_2 on the myocardium, inducing hyperkalemia, precipitating arrhythmias, and slowing the heart rate (20). Hypoxia is not a primary mechanism of action of death produced by CO_2 exposure, but rather it is the direct action of CO_2 on vital systems that produces unconsciousness and death.

Although hypoxia is not the means by which CO_2 produces death, the resistance of neonates to hypoxia and hypercarbia provides an explanation of why altricial neonates (those that are relatively undeveloped at birth) show prolonged survival times when exposed to CO_2. Altricial neonates have both long-term adaptations to hypoxia, which are a result of the extremely low oxygen partial pressure in the uterus (equivalent to an altitude of 6000 to 8000 m), and short-term responses to hypoxia and hypercarbia that resemble the responses of diving, burrowing, and hibernating mammals. In a review of these mechanisms, Singer discusses hypoxia tolerance in very young animals and compares it with the various hypoxia–hypercarbia adaptation schemes of several species (25). One long-term mechanism often discussed in the adaptation of neonates to hypoxia is the increased affinity of fetal hemoglobin for oxygen. At birth, however, fetal hemoglobin is absent in the mouse, so it is unlikely that this is a factor in the resistance of newborn mice to the effects of CO_2 (5, 6). Other factors, however, such as the decreased metabolic rate of neonates and the resistance of the neonatal brain to damage by hypoxia, probably do help to protect the preweanling mouse from the effects of CO_2 exposure (14). These innate neonatal tolerance mechanisms are best considered as a level of background resistance, not protective responses to a single hypoxic event. Acute protective responses in the neonate to severe hypoxia–hypercarbia include hypothermia, reduction of heart and respiration rate (the diving reflex), and reduction of blood pH. All the mechanisms of action of CO_2 on animals are compensated for by the newborn's normal responses to hypoxic–hypercarbic stress, therefore better tolerated by newborns than adults. It has been reported that laboratory rodents with precocial young, such as guinea pigs, do not express a large difference in susceptibility to CO_2 in newborns when compared with adults. For example, Glass and coworkers described CO_2 as causing death in 4.5 min for a newborn guinea pig and 3 min for animals at 7 days of age and adult guinea pigs (10).

The observed difference in time to death between inbred and outbred preweanling mice that occurred was unexpected. Inbred mice, however, appear less mature at birth than outbred mice and develop more slowly from birth to eye-opening. Developmental events, such as the lifting of the ear flap from the head or the acquisition of fur, which may be used to determine the age of inbred litters between days 0 and 6, reliably occur a day earlier in outbred mice. The lack of maturity in inbred mice may result in the neonatal protective mechanisms remaining active longer in these animals.

The rate of delivery of CO_2 was chosen to minimize potential distress due to hypothermia and noise. Although more sophisticated CO_2 delivery systems and euthanasia chambers were available to the authors, a simple plastic bag was chosen for several reasons. The use of a plastic bag allowed for easy observation and manipulation of the mice without changing the atmosphere surrounding them. Many research facilities currently use this method to euthanize animals, and every facility can obtain plastic bags. Plastic bags are easily sealed completely and provide immediate evidence of euthanasia chamber failure if they deflate. Because of the extended exposure times required for neonates, the use of a plastic bag for the euthanasia of preweanlings frees up other, perhaps more sophisticated, equipment for its more efficient use with adults, as they require shorter CO_2 exposure periods.

In the present study, several observations and recommendations can be made with regard to the euthanasia of neonatal mice with CO_2. Spontaneous respiratory efforts put forth by neonates may result in unintended recovery from what was thought to be a successful euthanasia. Often, neonatal mice are euthanized in groups (litters, etc.) and the recovery of one animal in the group may stimulate the recovery of its neighbors. The mice used in these studies, regardless of age, appeared to be unconscious within 60 sec, echoing the findings of Forslid in electroencephalography studies of swine under 80% CO_2 anesthesia, and those of Cartner and coworkers, who found that 100% CO_2 provided the most rapid loss of cortical function when compared with other methods of euthanasia (4, 8). Animals were not instrumented in the present study, however. The recommendations made for euthanasia times for neonatal mice (Table 3) fall roughly into week-long divisions in the development of the neonatal mouse. Alternatively, facilities may want to make exposure time decisions less complicated and categorize animals into two age groups—preweanlings and adults—to determine necessary times for CO_2 exposure. In the euthanasia system we used, animals were exposed to 100% CO_2. In euthanasia systems where CO_2 is mixed with oxygen or air, these exposure times may need to be adjusted and will probably be longer.

Acknowledgments

We would like to acknowledge Brian Corning, Anthony Nicholson, Beverly Paigen, Marcille Ripperton, and William White for their invaluable comments during the preparation of this manuscript. We also acknowledge the help of Torsten Promney in translation from German to English.

References

1. **Avery, R. C. and J. M. Johlin.** 1932. Relative susceptibility of adult and young mice to asphyxiation. Proc. Soc. Exp. Biol. Med. **29:**1184-1186.
2. **AVMA Panel on Euthanasia.** 2000. 2000 Report of the AVMA Panel on Euthanasia. J. Am. Vet. Med. Assoc. **218:**669-696.
3. **Blackshaw, J. K., D. C. Fenwick, A. W. Beattie, and D. J. Allan.** 1988. The behaviour of chickens, mice and rats during euthanasia with chloroform, carbon dioxide and ether. Lab. Anim. **22:**67-75.
4. **Cartner, S. C., S. C. Barlow, and T. J. Ness.** 2003. Loss of cortical function in mice following decapitation, cervical dislocation, potassium chloride injection, and CO_2 inhalation. ACLAM Forum. ACLAM, Sanibel Harbor, Fla., 14 to 17 May 2003.
5. **Craig, M. L. and E. S. Russell.** 1963. Electrophoretic patterns of hemoglobin from fetal mice of different inbred strains. Science **142:**398-399.
6. **Craig, M. L. and E. S. Russell.** 1964. A developmental change in hemoglobins correlated with an embryonic red cell population in the mouse. Dev. Biol. **10:**191-201.

7. **Feldman, D. B. and B. N. Gupta.** 1976. Histopathologic changes in laboratory animals resulting from various methods of euthanasia. Lab. Anim. Sci. **26:**218-221.
8. **Forslid, A.** 1987. Transient neocortical, hippocampal and amygdaloid EEG silence induced by one minute inhalation of high concentration CO_2 in swine. Acta Physiol. Scand. **130:**1-10.
9. **Forslid, A. and Ö. Augustinsson.** 1988. Acidosis, hypoxia and stress hormone release in response to one-minute inhalation of 80% CO_2 in swine. Acta Physiol. Scand. **132:**223-231.
10. **Glass, H. G., F. F. Snyder, and E. Webster.** 1944. The rate of decline in resistance to anoxia of rabbits, dogs, and guinea pigs from the onset of viability to adult life. Am. J. Physiol. **140:**609-615.
11. **Hewett, T. A., M. S. Kovacs, J. E. Artwohl, and B. T. Bennett.** 1993. A comparison of euthanasia methods in rats, using carbon dioxide in prefilled and fixed flow rate filled chambers. Lab. Anim. Sci. **43:**579-582.
12. **Humane Society of the United States** 2002. Concerns about carbon dioxide use in euthanasia and anesthesia. [Online] Available at http://www.hsus.org/animals_in_research/pain_distress/concerns_about_carbon_dioxide_use_in_euthanasia_and_anesthesia/. Accessed 3/16/05.
13. **Jaksch, W.** 1981. Euthanasia of day-old male chicks in the poultry industry. Int. J. Study. Anim. Probl. **2:**203-213.
14. **Kabat, H.** 1940. The greater resistance of very young animals to arrest of the brain circulation. Am. J. Physiol. **130:**588-599.
15. **Kavlock, R. J., R. D. Short, Jr., and N. Chernoff.** 1987. Further evaluation of an in vivo teratology screen. Teratog. Carcinog. Mutagen. **7:**7-16.
16. **Klaunberg, B. A., J. O'Malley, T. Clark, and J. A. Davis.** 2004. Euthanasia of mouse fetuses and neonates. Contemp. Top. Lab. Anim. Sci. **43:**29-34.
17. **Meyer, J. S., F. Gotoh, and Y. Tazaki.** 1961. CO_2 narcosis. An experimental study. Neurology **11:**524-537.
18. **National Association for Biomedical Research (NABR).** 1999. NABR Update **20:**1.
19. **Petty, W. C. and T. S. Sulkowski.** 1971. CO_2 narcosis in the rat. 1. Effects on respiration and blood parameters. Aerosp. Med. **42:**547-552.
20. **Petty, W. C. and T. S. Sulkowski.** 1971. CO_2 narcosis in the rat. II. Effects on the ECG. Aerosp. Med. **42:**553-558.
21. **Pontén, U.** 1966. Consecutive acid-base changes in blood, brain tissue and cerebrospinal fluid during respiratory acidosis and baseosis. Acta Neurol. Scand. **42:**455-471.
22. **Pontén, U. and B. K. Siesjö.** 1966. Gradients of CO_2 tension in the brain. Acta Physiol. Scand. **67:**129-140.
23. **Raj, A. B., M. C. Leach, and D. B. Morton.** 2004. Carbon dioxide for euthanasia of laboratory animals. Comp. Med. **54:**470-471.
24. **Reiss, M. and F. Haurowitz.** 1929. Über das Verhalten junger and alter Tiere bei erstickung. Klin. Wochenschr. **8:**743-744.
25. **Singer, D.** 1999. Neonatal tolerance to hypoxia: a comparative-physiological approach. Comp. Biochem. Physiol. A Mol. Integr. Physiol. **123:**221-234.