Journal of the American Association for Laboratory Animal Science
Copyright 2023
by the American Association for Laboratory Animal Science

Vol 62, No 3
May 2023
Pages 274–279

Euthanasia of Neonatal Rats and Mice using Carbon Monoxide

Debra L Hickman[1,2,*]

Minimization of potential pain and distress of rodents undergoing euthanasia is a touchstone of veterinary clinical medicine. Evaluation of this issue in postweanling rodents has supported revisions to the AVMA (American Veterinary Medical Association) Guidelines on Euthanasia in 2020. However, relatively little information is available on humane aspects of anesthesia and euthanasia in neonatal mice and rats. These neonates are not reliably euthanized by exposure to commonly used inhalant anesthetic agents due to their physiologic adaptations to hypercapnic environments. Therefore, options such as prolonged inhalant anesthetic gas exposure, decapitation, or use of injectable anesthetics are recommended for neonates. All of these recommended methods have operational implications, ranging from reported job dissatisfaction by animal care staff to rigorous reporting requirements associated with the use of controlled substances. This lack of a euthanasia method that does not entail operational issues hampers the ability of veterinary professionals to provide appropriate guidance to scientists working with neonates. This study was designed to assess the effectiveness of carbon monoxide (CO) as an alternative euthanasia agent for mouse and rat pups on postnatal days (PND) 0 to 12. The study demonstrates that CO may be a potential alternative for preweanling mice and rats at PND6 or older but is not appropriate for neonates at PND5 or younger.

Abbreviations and acronyms: CO, carbon monoxide; PND, postnatal day

DOI: 10.30802/AALAS-JAALAS-22-000103

Introduction

Minimization of potential pain and distress of rodents undergoing euthanasia is a touchstone of veterinary clinical medicine. Substantial resources have been allocated to the evaluation of this question in postweanling rodents,[9,11,12] resulting in revisions to the AVMA Guidelines on Euthanasia in 2020.[2] However, relatively little information is available on the humane aspects of anesthesia and euthanasia of neonatal mice and rats. These young animals are not reliably euthanized by exposure to commonly used anesthetic agents due to their physiologic adaptations to hypercapnic environments.[6,7,8] Therefore, options such as prolonged gas exposure, decapitation, or use of injectable anesthetics are recommended for neonates. All of these recommended methods have operational implications, ranging from reportedly greater job dissatisfaction by animal care staff to the rigorous requirements associated with the use of controlled substances. This lack of a humane euthanasia method that does not involve operational constraints hampers the ability of veterinary professionals to provide appropriate guidance to scientists working with neonates. Thus, alternative methods are needed for euthanasia of preweanling, altricial rodents.

Carbon monoxide (CO) has been used to euthanize a wide variety of species (for example, poultry, piglets, dogs, cats, and mink).[1] It is a highly toxic, odorless, and colorless gas that kills via induction of hypoxia. When inhaled, CO binds to hemoglobin, forming a highly stable carboxyhemoglobin molecule that competes with oxygen in the blood for binding to the same heme group of hemoglobin. On initial exposure, people report headache and fatigue. With higher concentrations or prolonged exposure, loss of consciousness and cardiorespiratory arrest follow.[1] However, CO exposure is not associated with distress or pain. Homes and businesses are encouraged or mandated to maintain CO alarms that can alert inhabitants to the presence of toxic levels before unconsciousness occurs.[5] Initial assessment of the responses of rats to CO suggests that it is not highly aversive, especially as compared with carbon dioxide,[4] although findings in mice were less conclusive.[3]

The high toxicity of CO has led to reluctance to consider it as an alternative method for animal euthanasia. After lengthy conversations with colleagues in the Purdue University School of Engineering and representatives of the Indiana University Environmental Health and Safety (EHS) group regarding the possible use of CO, we determined that CO toxicityis a manageable risk. Our discussions indicated the value in determining whether low concentrations of CO can provide a safe, effective, and irreversible method of euthanasia for preweanling rodents. It should be noted that both carbon dioxide and isoflurane have potentially significant animal welfare and personnel safety concerns.

This study was designed to assess the effectiveness of CO as a euthanasia agent for preweanling rats and mice. We hypothesized that CO gas would be better than carbon dioxide and halogenated anesthetics in providing a humane euthanasia for altricial neonates because the binding of heme by CO was expected to cause consistently rapid death in these animals.

Materials and Methods

As reported previously for carbon dioxide,[67] we used the following procedures. Neonatal rats were placed in groups of 2 to 6 (depending on number of available neonates) into a clear glass container (pint canning jar [5 in. tall × 3 in. in diameter]; Ball Pint

Submitted: 08 Nov 2022. Revision requested: 07 Dec 2022. Accepted: 13 Mar 2023.
[1]College of Veterinary Medicine, Department of Comparative Pathobiology, Purdue University, West Lafayette, Indiana; [2]Laboratory Animal Resource Center, School of Medicine, Indiana University, Indianapolis, Indiana
**Corresponding Author. Email: hickmand@purdue.edu*

Figure 1. Experimental setup including clear glass pint jar with lid and CO meter.

Mason Jars, Ball, Broomfield, CO) with a lid modified to allow placement of a 0.5-in. plastic tube and two 2-mm (about 0.08-in.) holes to prevent over-pressurization of the container (Figure 1). CO (8% in room air; custom blend by Praxair, Indianapolis, IN) flowed continuously into the container at 0.75 L/min. To place the neonates into the jar, they were placed just within the neck of the jar, then the jar was gently tipped to arrange the neonates so that they were dispersed along the glass, with the jar laying on its side. The concentration of 8% was selected based on review of the literature, which indicated that concentrations of 6 to 12% were used for the euthanasia of other species.[1] These procedures were repeated for neonatal mice, which were also placed in the euthanasia container in groups of 2 to 6. The experimental unit was each individual neonate. All activities were performed in a fume hood with CO sensors placed at the sash for the protection of personnel. If a sensor was triggered, the CO was immediately turned off and the space vacated until the alarm stopped. At that point, the apparatus was assessed to ensure that connections were set up appropriately.

The neonates were assessed visually for movement, apparent consciousness, and respiratory activity to ensure that death had occurred prior to their removal from the container. After removal from the container, the neonates were placed on a 37 °C surface and monitored for 20 min for signs of recovery from CO exposure such as breathing, return of pink color, or movement. Neonates were initially exposed to CO for 5 min. Any neonate that recovered was immediately euthanized by decapitation with sharp scissors. If any neonate from any age group recovered, the trial was scored as a fail, and the CO exposure period was lengthened by 30 to 60 s, and another group of that age was tested. If no animals recovered, the trial was scored as a pass, and that duration treatment was repeated until 40 neonates had been successfully euthanized with this exposure time to the CO. Exposure times ranged from a minimum of 5 min to a maximum of 30 min. We assessed neonatal rats and mice at 13 ages, ranging from the day of birth (PND0) to 12 days after birth (PND12).

Experimental design. When an exposure time was identified that resulted in death of 40 neonates of a particular age group, 2 more exposure times (at least 30 to 60 s longer than the initial successful exposure time) were assessed to identify the useful range of exposure times to ensure death for that age of neonate. For example, if 5 min of CO exposure resulted in the death of 40 neonatal mice, 5.5 and 6 min were also assessed for an additional 40 neonates for each exposure time. If all 40 neonates were also died after these exposure times, data collection was stopped, and the appropriate exposure time was noted as 5 to 6 min.

Animals. Care was taken to distribute the different stocks, strains, and ages across all exposure times to avoid inappropriate weighting of any given exposure time. To accomplish this, each cage of rats or mice used from a given colony and age would be assigned a different exposure time until all times had representation from that colony.

To reduce the overall number of animals used on this study, we partnered with our researchers to establish breeder pairs from animals that were scheduled for euthanasia and to use their offspring. Although using surplus animals from our breeding colonies would have been preferable, the generation of transgenic mice does not facilitate this option. Because genotyping typically does not occur until PND 10 to 14, most "surplus" mice (for example, those of the undesirable genotype) would be too old for use in this study. Therefore, we had to generate most of the litters to obtain pups of the correct age. However, over 70% of the breeding colonies on campus were managed by the animal care staff. This gave us a unique opportunity to refine our use of animals. We worked closely with the breeding colony management team to identify and divert neonatal mice scheduled for euthanasia before 12 d of age (for example, unintended litters) whenever possible to reduce the overall number of animals produced specifically for this study. We were able to obtain approximately 25% of the animals used for this study through this collaboration.

The breeding mice were pair-housed in IVC (Alt Design; Siloam Springs, AR) with corncob bedding (Bed-O-Cobs; The Andersons, Maumee, OH) and nesting materials (Enviro-Dri; Shepherd Specialty Papers, Milford, NJ). The mouse colonies were screened quarterly using indirect sentinels. At the time of the study, the colonies were free of the following pathogens: Sendai virus, pneumonia virus of mice, mouse coronavirus, parvoviruses (mouse parvovirus 1 and 2 and mouse minute virus), murine rotavirus (EDIM), mouse adenovirus 1 and 2, ectromelia, GDVII, reovirus, lymphocytic choriomeningitis virus, *Clostridium piliforme*, *Mycoplasma pulmonis*, pinworms (*Aspicularis tetraptera*, *Syphacia* spp.), and fur mites (*Radfordia ensifer*, *Ornithonyssus bacoti*).

The breeding rats were pair-housed in IVC (Alt Design, Siloam Springs, AR) with hardwood bedding (Sani-Chip; PJ Murphy Forest Products, Montville, NJ) and wood gnawing blocks (BioServ, Fleminton, NJ). The rat colonies were also screened quarterly using indirect sentinels. At the time of the study, the colonies were free of the following pathogens: coronavirus (sialodacryoadenitis virus), parvoviruses (NS1, rat pneumonia virus, Kilham rat virus, H1 virus, rat minute virus), theliovirus, *Clostridium piliforme*, *Mycoplasma pulmonis*,

pinworms (*Aspicularis tetraptera*, *Syphacia* spp.), and fur mites (*Radfordia ensifer*, *Ornithonyssus bacoti*).

All rodents received food (Teklad 2018×; Envigo, Indianapolis, IN) free choice, with reverse-osmosis water provided through an automatic watering system. Cages were changed at least every other week for mice and at least weekly for rats in a laminar flow workstation (Nuaire, Plymouth, MN) and were autoclaved prior to reuse. Hands and implements were disinfected with Rescue (Quip Labs, Wilmington, DE) between cages. The macroenvironment included a 12:12-h light:dark cycle (lights on, 0700), temperature of 72 ± 1 °F (22 ± 1 °C), and humidity between 30% and 70%.

Statistical analyses were not performed for this study as the goal was to assess the success or failure of various exposure times to CO to cause the death of preweanling rats and mice.

All studies were performed at Indiana University School of Medicine and were approved by the Indiana University School of Medicine IACUC before the start of the study. This program is accredited by AAALAC, International and compliant with all applicable federal regulations.

Results

From a safety perspective, we considered the use of CO for euthanasia to be a manageable risk for personnel. When we used a 0.75-L/min flow rate, CO never reached the concentrations necessary to trigger sensors during use of the system, including a sensor placed at the exhaust holes in the lid of the euthanasia chamber.

A total of 1081 neonatal mice were used for this study. Over half of the mice recovered from CO exposure for 30 min at PND5 (20 neonates), PND4 (9 neonates), PND2 (9 neonates), and PND1 (10 neonates). Therefore, PND3 and PND0 neonates were not tested (Table 1). This refinement allowed us to bypass exposure times that were highly likely to permit recovery.

Table 1. Range of exposure times that resulted in the deaths of 120 of 120 (100%) of neonatal mice of the listed age. Mice and rats were assessed 20 min after removal from the carbon monoxide. Forty neonates of each species were tested at 30-s (indicated by *) or 60-s intervals to obtain 40 animals at each of the 3 time points in the range.

Age	CO time range for mice	CO time range for rats
PN0	> 30 min (presumed)	> 30 min (presumed)
PN1	> 30 min	> 30 min (presumed)
PN2	> 30 min	> 30 min (presumed)
PN3	> 30 min (presumed)	> 30 min
PN4	> 30 min	> 30 min
PN5	> 30 min	> 30 min
PN6	20 to 22 min	21 to 23 min
PN7	14 to 16 min	17 to 19 min
PN8	8 to 10 min	12 to 14 min
PN9	8 to 10 min	13 to 15 min*
PN10	7 to 8 min*	9 to 10 min*
PN11	7 to 8 min*	8 to 9 min*
PN12	5 to 6 min*	6.5 to 7.5 min*

For PND6, 10 mouse pups were tested with 15 min of exposure (90% died), and 40 mouse pups each were tested with 20, 21, and 22 min of exposure (100% died). For PND7, 11 mouse pups were tested with 10 min of exposure (100% died), 12 with 11 min (91% died), 11 with 12 min (100% died), and 4 with 13 min (75% died). Forty mouse pups each were tested with 14, 15, and 16 min of exposure, which resulted in 100% deaths. The percentage of mice that died for PND8 to PND12 and the total number tested are shown in Table 2.

Between PND6 and 9, mice required exposure times that ranged from 10 to 20 min to reliably produce death (Table 2). Mice that were older than PND10 required exposure times of between 5 and 8 min to produce death (Table 2).

A total of 1459 neonatal rats were used for this study. Over 50% recovered after 30 min of exposure time for PND5 (65 neonates), PND4 (16 neonates), and PND3 (2 neonates). PND0 through 2 were not tested, thus omitting exposure times that were not likely to cause death.

For PND6, 4 rat pups were tested with 15 min of exposure (100% died), 19 with 17 min (89% died), 14 with 18 min (14% died), 9 with 19 min (9% died), and 33 with 20 min (90% died). Forty PND6 rat pups each were tested at 21, 22, and 23 min of exposure, all with 100% death. For PND7, 29 rat pups were tested with 10 min of exposure (100% died), 16 with 11 min (100% died), 20 with 12 min (100% died), 30 with 13 min (96% died), 13 with 14 min (100% died), 32 with 15 min (96% died), and 31 with 16 min (90% died). Forty PND7 pups each were tested for 17, 18, and 19 min of exposure with 100% death. The percentage of rats that died between ages of PND8 to PND12 is presented in Table 3, with the total number of rats tested in parentheses.

Rats of ages that were between PND6 and PND9 required exposure times that ranged from 13 min to 23 min to reliably induce death (Table 1). Rats that were older than PND10 required exposure times of between 6.5 to 10 min to cause death (Table 1).

Discussion

This study supported the findings of previous studies suggesting that administration of inhalant anesthetics and other compounds does not reliably kill neonatal rats and mice after 30 min of exposure to the gas.[6,7,8] This lack of efficacy may occur because altricial rodents of this age are highly resistant to hypoxic environments like those present in the uterus.[10] Previous reviews have described the mechanisms that underly this tolerance for hypoxic environments, such as the low metabolic rate of neonates and innate resistance of the brain to hypoxic damage.[2,10] Therefore, methods of euthanasia such as barbiturate administration via the intraperitoneal route or decapitation have been recommended for neonatal mice and rats that are PND5 or younger.[2]

Considering the data presented in previous studies,[6,7] CO may be slightly advantageous over carbon dioxide in mice, as CO seems to cause death in older neonates after a shorter exposure

Table 2. Percentage of neonatal mice that died, with the total number of mice tested in parentheses.

	5 min	5.5 min	6 min	6.5 min	7 min	7.5 min	8 min	9 min	10 min
PN8	0 (4)		0 (4)		92 (40)		100 (40)	100 (40)	100 (40)
PN9	50 (8)		0 (4)		62 (8)		100 (40)	100 (40)	100 (40)
PN10	95 (40)	100 (40)	100 (40)	92 (12)	100 (40)	100 (40)	100 (40)		
PN11	76 (13)	60 (5)	74 (27)		100 (40)	100 (40)	100 (40)		
PN12	100 (40)	100 (40)	100 (40)						

Table 3. Percentage of neonatal rats that died, with the total number of rats tested in parentheses. Yellow shading represents the 3 groups of 40 neonates that were successfully euthanized. Gray shading represents a test that was not done.

	5 min	5.5 min	6 min	6.5 min	7 min	7.5 min	8 min	8.5 min	9 min	9.5 min	10 min	11 min	12 min	13 min	14 min	15 min
PN8	ND	ND	ND	ND	ND	ND	100 (4)	ND	100 (5)	ND	36 (11)	0 (7)	100 (40)	100 (40)	100 (40)	
PN9	0 (5)	ND	ND	ND	100 (5)	ND	50 (8)	ND	100 (5)	ND	90 (10)	100 (14)	90 (10)	100 (40)	100 (40)	100 (40)
PN10	100 (8)	75 (4)	63 (8)	88 (16)	100 (24)	100 (18)	96 (27)	91 (11)	100 (40)	100 (40)	100 (40)					
PN11	ND	ND	78 (9)	85 (20)	100 (24)	90 (20)	100 (40)	100 (40)	100 (40)							
PN12	100 (40)	100 (24)	97 (32)	100 (40)	100 (40)	100 (40)										

Table 4. Table modified from previous studies[6,7] to allow comparison of carbon monoxide and carbon dioxide minimal exposure times to ensure death in neonatal mice and rats

Age of mouse	Carbon monoxide minimal exposure time	Carbon dioxide minimal exposure time
PN0 to PN6	> 30 min (not recommended)	60 min (not recommended)
(PN7 to PN13	16 min	20 min
Age of rat	Carbon monoxide minimal exposure time	Carbon dioxide minimal exposure time
PN0 to PN6	> 30 min (not recommended)	40 min (not recommended)
PN7 to PN13	20 min	20 min

time. However, this difference may not apply to neonatal rats (Table 4).

Although the use of CO can entail significant occupational health and safety risks, we managed these risks by using low concentrations of CO in well-ventilated spaces. This finding is consistent with previous use of CO as an approved method of euthanasia for multiple species.[2]

Our study confirms previous reports of the difficulty of using inhalant agents to euthanize very young neonatal mice and rats (PND5 or younger).[6,7,8] However, as postnatal development continues, inhalant agents become more effective for the euthanasia of preweanling rodents.

Acknowledgments

This study was supported by a grant from the American College of Laboratory Animal Medicine (ACLAM) Foundation. A portion of the mice used for this study were obtained in collaboration from the NIH/NCI Cancer Center Support Grant (P30 CA082709)

References

1. **Leary S, Underwood W, Anthony R, Cartner SC, Grandin T, Greenacre C, Gwaltney-Brant S, McCrackin M, Meyer R, Miller D, Shearer J, Turner T, Yanong R.** [Internet]. 2020. AVMA guidelines for the euthanasia of animals: 2020 edition. [Cited 10 May 2021]. Available at: https://www.avma.org/sites/default/files/2020-01/2020-Euthanasia-Final-1-17-20.pdf.
2. **Li S, Hafeez A, Noorulla F, Geng X, Shao G, Ren C, Lu G, Zhao H, Ding Y, Ji X.** 2017. Preconditioning in neuroprotection: From hypoxia to ischemia. Prog Neurobiol **157**:79–91. https://doi.org/10.1016/j.pneurobio.2017.01.001.
3. **Makowska IJ, Vickers L, Mancell J, Weary DM.** 2009. Evaluating methods of gas euthanasia for laboratory mice. Appl Anim Behav Sci **121**:230–235. https://doi.org/10.1016/j.applanim.2009.10.001.
4. **Makowska IJ, Weary DM.** 2009. Rat aversion to carbon monoxide. Appl Anim Behav Sci **121**:148–151. https://doi.org/10.1016/j.applanim.2009.08.008.
5. **National Fire Protection Association.** [Internet]. 2008. Carbon monoxide. [Cited 3 March 2018]. Available at: https://www.nfpa.org/Public-Education/By-topic/Fire-and-life-safety-equipment/Carbon-monoxide.
6. **Pritchett K, Corrow D, Stockwell J, Smith A.** 2005. Euthanasia of neonatal mice with carbon dioxide. Comp Med **55**:275–281.
7. **Pritchett-Corning KR.** 2009. Euthanasia of neonatal rats with carbon dioxide. J Am Assoc Lab Anim Sci **48**:23–27.
8. **Seymour TL, Nagamine CM.** 2016. Evaluation of isoflurane overdose for euthanasia of neonatal mice. J Am Assoc Lab Anim Sci **55**:321–323.
9. **Shomer NH, Allen-Worthington KH, Hickman DL, Jonnalagadda M, Newsome JT, Slate AR, Valentine H, Williams AM, Wilkinson M.** 2020. Review of rodent euthanasia methods. J Am Assoc Lab Anim Sci **59**:242–253. https://doi.org/10.30802/AALAS-JAALAS-19-000084.

10. **Singer D.** 1999. Neonatal tolerance to hypoxia: A comparative-physiological approach. Comp Biochem Physiol A Mol Integr Physiol **123**:221–234. https://doi.org/10.1016/S1095-6433(99)00057-4.
11. **Steiner AR, Flammer SA, Beausoleil NJ, Berg C, Bettschart-Wolfensberger R, Pinillos RG, Golledge HDW, Marahrens M, Meyer R, Schnitzer T, Toscano MJ, Turner PV, Weary DM, Gent TC.** 2019. Humanely ending the life of animals: Research priorities to identify alternatives to carbon dioxide. Animals (Basel) **9**:911. https://doi.org/10.3390/ani9110911.
12. **Turner PV, Hickman DL, van Luijk J, Ritskes-Hoitinga M, Sargeant JM, Kurosawa TM, Agui T, Baumans V, Choi WS, Choi YK, Flecknell PA, Lee BH, Otaegui PJ, Pritchett-Corning KR, Shimada K.** 2020. Welfare impact of carbon dioxide euthanasia on laboratory mice and rats: A systematic review. Front Vet Sci **7**:411. https://doi.org/10.3389/fvets.2020.00411.