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Evaluation of Isoflurane Overdose for Euthanasia of Neonatal Mice

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Neonatal mice (that is, pups younger than 6 d) must be exposed to CO_2 for as long as 50 min to achieve euthanasia. Alternatively, other inhalant anesthetic agents have been used to euthanize laboratory rodent species. We investigated the efficacy of isoflurane at saturated vapor pressure to euthanize neonatal mice. Neonatal mice (*n* = 76; age, 1 or 2 d) were exposed to isoflurane in a sealed, quart-size (0.95-L) plastic bag at room temperature. Righting and withdrawal reflexes were absent in less than 2 min. After 30 min of exposure to isoflurane, pups were removed and monitored for recovery. All pups were cyanotic and showed no detectable signs of life when they were removed from the bag. However, after 30 to 120 min after removal from the bag, 24% of isoflurane-overexposed pups began gasping and then resumed normal respiration and regained a normal pink coloration. These results demonstrate that isoflurane overexposure at saturated vapor pressure for 30 min is insufficient to euthanize neonatal mice and that isoflurane overexposure must be followed by a secondary means of euthanasia.

Euthanasia methods require ongoing research and refinement in the laboratory animal environment. Euthanasia methods must be practical, quick, effective, and above all, humane. Human safety and perception are of utmost importance also.[2] The euthanasia of neonatal mice (defined as pups 6 d of age or younger), in particular, is a significant challenge in modern laboratory animal research facilities. Carbon dioxide, the most common anesthetic agent used for euthanasia in laboratory rodents, can take as long as 50 min to euthanize a neonatal mouse.[14] This prolonged exposure is in part due to the well-documented tolerance of neonates to hypoxia.[5,15] In addition, the development of nociceptive pathways is incomplete during the first 2 wk of life.[1,7,10] Carbon dioxide euthanasia presents disadvantages in adult rodents as well, most notably an aversion to CO_2 which can cause appreciable distress prior to loss of consciousness.[11,12,17] These complications have led researchers to use other gas anesthetics to euthanize laboratory rodents.

Isoflurane is a common inhalant anesthetic in laboratory animal medicine and veterinary practice. Although its exact mechanism of action is complex and not fully understood, the primary anesthetic effect of isoflurane involves activation of γ-aminobutyric acid channels and a subsequent increase in the chloride permeability of neurons.[4,6] The AVMA guidelines list inhaled anesthetics (halothane, isoflurane, sevoflurane, and desflurane with or without nitrous oxide) as "acceptable with conditions" as methods of euthanasia for neonatal laboratory rodents.[2] However, a previous study recommended against the use of inhalation anesthetic overdose for the euthanasia of neonatal rodents because of the inconsistent and delayed onset of cardiac arrest.[9] However, the cited study[9] used an anesthetic vaporizer set to its maximum level (5% volume of atmosphere). To our knowledge, no studies have investigated the time required for euthanasia of neonatal mice through isoflurane inhalation under saturated vapor pressure at room temperature (approximately 31% at 760 mm Hg and 20 °C[16]). Here we further characterized isoflurane's use as a euthanasia agent for neonatal mice. We hypothesized that isoflurane used at saturated vapor pressure would be more efficacious than CO_2 in this context. The current study was designed to offer practical recommendations regarding the euthanasia of neonatal mice by isoflurane overdose.

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Materials and Methods

Animals. All animals were used in accordance with the *Guide for the Care and Use of Laboratory Animals*.[8] The study population comprised male and female neonatal mice (*n* = 76; age, 1 or 2 d) with C57BL/6 and CD1 genetic backgrounds that were scheduled for euthanasia. Mice were housed in an AAALAC-accredited Stanford University vivarium. Experiments were conducted with the approval of Stanford University's Administrative Panel on Laboratory Animal Care. Mice were maintained in disposable caging (Innovive, San Diego, CA) with biweekly cage changes. Animals in this study were housed within a barrier facility; health surveillance was performed through quarterly testing of dirty-bedding CD1 sentinels (Charles River Laboratories, Hollister, CA). Sentinels were consistently negative for mouse parvovirus, minute virus of mice, mouse hepatitis virus, mouse rotavirus, murine encephalomyelitis virus, murine norovirus, Sendai virus, mouse adenovirus 1 and 2, ectromelia virus, lymphocytic choriomeningitis virus, pneumonia virus of mice, reovirus 3, *Mycoplasma pulmonis*, *Helicobacter* spp., *Pasteurella pneumotropica*, and endo- and ectoparasites.

Isoflurane euthanasia. Groups of neonatal mice (7 groups, 3 to 18 mice per group) were placed into clear, plastic, sealable, quart-size storage bags that each held an approximately 5 cm^2 piece of absorbent material (paper towel or compacted cotton fibers [Nestlets, Ancare, Bellmore, NY]; Figure 1). Immediately after the pups were added to the bag, 0.5 mL of isoflurane (Henry Schein Animal Health, Dublin, OH) was applied to the absorbent material. The isoflurane liquid was immediately and completely adsorbed, precluding any free isoflurane liquid in the bag and resulting in a saturated vapor pressure

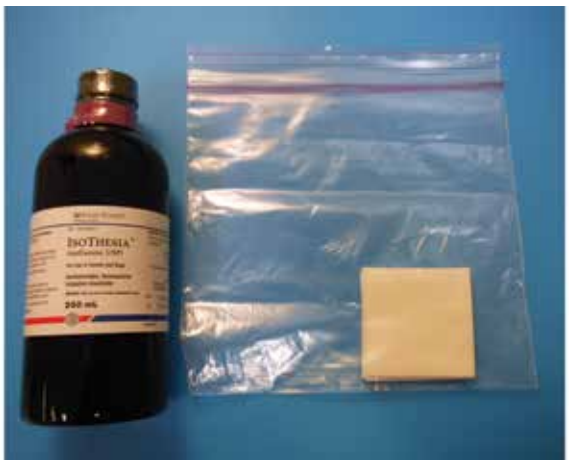

Figure 1. Materials used for the euthanasia of neonatal mice by isoflurane overexposure. Immediately after the pups were added to the bag, isoflurane (0.5 mL) was placed onto the absorbent paper by using a transfer pipette, and the bag was sealed.

of approximately 31%.[16] To minimize stress, the bag always contained a variable amount of trapped air (no attempt was made to remove the air), and the pups had sufficient room to be recumbent without contacting adjacent littermates or the absorbent material. The pups were continuously monitored for respiration, color, and movement. Righting reflex was monitored by tilting the bag to cause the pups to be on their backs. The withdrawal reflex was checked by gently pressing on the tail or paw of the pups through the plastic bag. The bag was kept tightly closed in a cage-change or fume hood in a room with nonrecirculating ventilation. After 30 min, the pups were removed from the bag, placed on a paper towel at room temperature, and monitored every 5 min for as long as 120 min. Pups were kept at room temperature to mimic typical euthanasia conditions. Recovery was defined as the neonate regaining spontaneous, regular respiration; voluntary movement; and a healthy, pink skin color. Euthanasia was confirmed in all animals by decapitation immediately after the experiment.

Results

Respiration ceased and the righting and withdrawal reflexes were absent within 2 min after isoflurane was introduced into the bag. After the 30-min exposure, the pups were cyanotic, with no observable signs of life. Surprisingly, after a period of time ranging from 30 to 120 min after removal from the bag, 18 of the 76 (23.7%) mice began gasping and then resumed normal respiration, and the cyanotic skin regained a normal pink coloration (Table 1, Figure 2). Of the 18 pups that recovered after isoflurane overexposure, 14 (77.8%) did so within 60 min after removal from the bag. However, 2 pups recovered at 71 and 90 min after removal from the bag, and an additional 2 pups recovered as late as 120 min after removal from the bag, at which time the experiment was terminated.

Discussion

A review of the literature provided limited information on the euthanasia of neonatal mice. Two studies assessing CO_2 euthanasia in neonatal mice[14] and rats[13] demonstrated the need for prolonged exposure times to euthanize neonatal rodents compared with adults (recommended times: neonatal mice, 60 min; adult, 5 min;[14] neonatal rats, 40 min; adult rats, 5 min[13]). The 2013 AVMA euthanasia guidelines cites those studies[13,14] and recommends "adequate exposure time" for the euthanasia of rodent fetuses and neonates by inhaled anesthetic overdose.[2] In addition, within an undisturbed euthanasia chamber covered by a lid with two 1-cm holes, CO_2 levels had decreased to 10% to 20% by 20 min after the CO_2 was turned off.[3] Therefore ensuring that the CO_2 concentration in similarly constructed chambers is maintained at levels appropriate for euthanasia for longer than 60 min requires that the CO_2 gas remains on for an extended period of time.

The goal of the current study was to test the hypothesis that, compared with CO_2, isoflurane administered at saturated vapor pressure to neonatal mice would be more efficacious for euthanasia and therefore a refinement regarding the euthanasia of neonatal mice and possibly other rodents. Although rodents display aversion to all inhalant anesthetics, isoflurane reportedly causes less aversion in mice[11] and rats[11,17] initially than does CO_2. Isoflurane is readily available, inexpensive, and does not require additional equipment for this application. However, working with isoflurane poses an occupational health and safety risk. To reduce the risk of human exposure by inhalation, we recommend the use of sufficient ventilation or a chemical fume hood when working with isoflurane in this manner. Our data show that although isoflurane overdose quickly rendered neonatal mice unconscious within 2 min, 30 min of exposure was insufficient to ensure that all pups died. We conclude that exposing neonatal mice to isoflurane at saturated vapor pressure for 30 min as a means of euthanasia must be followed by a secondary method (for example, decapitation) to ensure a humane death.

Table 1. Number and percentage of neonatal mice that recovered after 30 min of isoflurane overexposure

Experiment	Age (d)	No. of pups		Percentage (%) recovered
		Total	Recovered	
1	1	18	2	11
2	1	16	3	20
3	1	3	0	0
4	1	10	6	60
5	1	10	2	20
6	2	11	2	18
7	2	8	3	38

Overall, 18 of 76 (24%) of mouse pups recovered.

Figure 2. A recovered pup (*) demonstrating its pink coloration among its cyanotic littermates.

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