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Testing three measures of mouse insensibility following induction with isoflurane or carbon dioxide gas for a more humane euthanasia

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ABSTRACT

Laboratory mice are commonly killed via exposure to gradually increasing concentrations of isoflurane and carbon dioxide (CO_2) gas. Once rendered insensible using isoflurane or CO_2, a high concentration of CO_2 is used to decrease time to death. When the switch from isoflurane to a high flow rate of CO_2 is made, it is important that the mice are insensible (complete loss of sensation) to prevent conscious exposure to potentially painful and distressing CO_2 concentrations (>40%). Currently, no science-based methods exist for users to know when insensibility occurs during isoflurane or CO_2 euthanasia protocols. The objective of our study was to examine three measures of insensibility: recumbency onset, loss of the righting reflex and loss of the pedal withdrawal reflex, in mice during euthanasia with 20% gradual-fill CO_2 ($n = 6$) or 5% isoflurane using 2 L/min oxygen as the carrier gas, followed by CO_2 ($n = 7$). In addition to testing for the three insensibility indicators, an 'escape response' was recorded if a mouse exhibited leg paddling and forward or lateral movement in response to the experimenter's approaching hand, when about to test for loss of the righting reflex. A 'purposeful movement' response was recorded if the animal showed the inability to self-right, but exhibited leg paddling while on its back. The results of this study demonstrated that all isoflurane treatment mice showed an escape response, purposeful movement and a pedal withdrawal response, in comparison to only two, zero and one CO_2 mice, respectively. Thus, even after recumbency and loss of the righting reflex, mice showed indications of sensibility, suggesting that these are not reliable indicators of insensibility when using the isoflurane method of euthanasia as outlined in this study. On average (±S.D.) the interval between recumbency onset and loss of the pedal withdrawal reflex was 40 (±13) s for isoflurane versus 16 (±11) s for the CO_2 method tested. When using isoflurane induction as described here, we recommend that users wait a minimum of 79 s (mean + 3 S.D.) after the appearance of recumbency before switching to a high flow rate of CO_2. If using the gradual-fill CO_2 method as described here, users should wait a minimum of 49 s (mean + 3 S.D.) before increasing the flow rate of CO_2.

1. Introduction

Laboratory rodents are commonly euthanized via exposure to gradually increasing concentrations of isoflurane or CO_2 gas. However, mice and rats find CO_2 gas aversive and are unwilling to tolerate exposure at concentrations

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sufficient to cause insensibility (complete loss of sensation) (Kirkden et al., 2008; Krohn et al., 2003; Leach et al., 2002a; Makowska et al., 2009; Moody and Weary, 2014; Niel et al., 2008; Niel and Weary, 2007; Wong et al., 2013). Recent evidence shows that isoflurane is less aversive to mice and rats than CO_2 (Leach et al., 2002b, 2004; Makowska and Weary, 2009; Moody and Weary, 2014; Wong et al., 2013) and other inhalant anesthetics (Makowska et al., 2009). Isoflurane is used first to induce insensibility; once the animals are insensible, isoflurane can be turned off and immediately switched to a high flow rate of CO_2 for more rapid killing than is possible with isoflurane alone.

It is important that the mice are insensible when the switch from isoflurane to CO_2 is made, given that CO_2 concentrations greater than 3% are aversive to mice and rats (as cited above), concentrations greater than 10% CO_2 cause fear responses in mice and rats (Concas et al., 1993; Niel and Weary, 2006; Ziemann et al., 2009) and act as an unconditioned fear stimulus in mice (Ziemann et al., 2009) and that concentrations >40% cause pain due to the conversion of CO_2 into carbonic acid on mucosal surfaces (Anton et al., 1992; Thurauf et al., 2002).

Currently, no scientifically established measures exist for users to practically and reliably know when loss of complete sensibility occurs in rodents induced with isoflurane as part of a euthanasia protocol. This is problematic because animals should be completely insensible before exposure to CO_2, especially high concentrations (>40%) used for more rapid killing. One recent study by Valentine et al. (2012) reported that five of 10 mice anesthetized with isoflurane re-gained sensibility after isoflurane was switched to CO_2; recovery after the switch represents the worst-case scenario as animals experience the negative effects of isoflurane induction and exposure to high concentrations of CO_2. One reason why animals may recover during this procedure is that the induction period with isoflurane was insufficient to produce insensibility, therefore animals react when exposed to aversive concentrations of CO_2.

The aim of the current experiment was to evaluate three progressive measures of insensibility (onset of recumbency, loss of the righting reflex and loss of the pedal withdrawal reflex; Table 1) to establish when to switch from isoflurane to a high flow rate of CO_2 gas when euthanizing laboratory mice. It was hypothesized that insensibility occurs sometime after the onset of recumbency. For comparison, we also examined these same response measures for mice exposed to gradual-fill CO_2, as some laboratories continue to use the agent for induction and then increase the flow rate when animals are thought to be insensible to decrease time to death.

Table 1
Definitions for the behavioral indicators of insensibility applied to mice undergoing the isoflurane or CO_2 method of euthanasia.

Behavior	Definition
Recumbency onset	Head resting on cage floor, head and body motionless, loss of muscle tone
Loss of righting reflex	Unable to self-right when placed on back
Loss of pedal withdrawal reflex	The first of three consecutive non-responses to alternating hind paw toe pinches, applied every 10 s between the metatarsal and phalanges bone

2. Materials and methods

2.1. Animals and housing

We used 13 surplus C57BL6/J male mice designated for euthanasia by The Centre for Disease Modeling at the University of British Columbia, Vancouver, Canada. Mice were housed under a 12 h light:12 h (lights off at 19:00 h) dark cycle, weighed between 28 and 34 g and were 3–4 months old. Testing occurred between 12:30 and 15:00 h during the light cycle. Mice were housed in groups of four or five in ventilated polysulfone type II long cages (Bioscape, Germany) (20.7 cm length × 14.0 cm width × 36.5 cm height) using an individually ventilated cage system (Bio A. S.® cage rack and blower system, Bioscape, Germany) complete with corncob bedding (7087 Soft Cob Bedding, Harlan Teklad, Madison, WI, USA), a transparent red-tinted polycarbonate nest box (Mouse Igloo, Bio-Serve, NJ, USA) (5.7 cm height × 10.5 cm width), brown crinkle paper (Enviro-dri, Shepherd Specialty Papers Inc., Richland, MI, USA) and one cotton nest square (Ancare, Bellmore, NY, USA) per cage. All animals were given *ad libitum* access to irradiated food (Global Rodent Diet 2918, Harlan Teklad, Madison, WI, USA) and reverse osmosis water in autoclaved 250 mL water bottles (Bioscape, Germany). Average humidity and temperature during testing were 62% and 21.2 °C, respectively. The University of British Columbia's Animal Care Committee approved all animal procedures used during this study and all mice were specific pathogen free of commonly tested viral and bacterial pathogens and parasites.

2.2. Experimental apparatus

The test cage (Fig. 1) consisted of a transparent, disposable individually ventilated mouse cage (Innocage® Universal Euro Type II Long, Innovive Inc., San Diego, CA, USA) (37.3 cm length × 23.4 cm width × 14 cm height) in which we cut a hole (6.5 cm diameter) in one side to project a surgical nitrile glove (Kimtech Pure G3, Kimberly-Clark Professional, Roswell, GA, USA, 30.5 cm, Size 7) sealed using a duct tape. This allowed one hand to be placed into the cage during testing. A non-slip pad made from wood floor

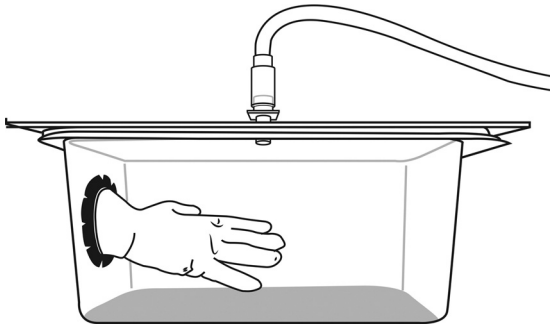

Fig. 1. Diagram of the experimental apparatus.